REMARKS

Upon entry of these amendments, claims 87, 89-98 will be pending in the instant application. Claim 88 has been cancelled. Support for amendments in claims 87, 89, 91, and 97 are at page 10, lines 12-16 and 32-36. Amended claims 87 and 97 now require the egg recipient and the somatic cell nucleus be the same species, the scope of which is commensurate with the scope of the teachings provided by the specification as indicated by the Examiner. (see Office Action page 7). Support for amended claim 97 and new claim 98 is found at page 58, lines 20-34 of the specification.

Thus, no new matter has been added by this amendment.

Double Patenting

Claims 87-96 have been rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-5 of US Patent No. 5,480,772. In response, Applicant submits herewith a terminal disclaimer along with the appropriate fee. Thus, this rejection has been overcome and should be withdrawn.

Claims 87-96 have also been rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-14 of US Patent No. 5,651,992. In response, Applicant submits herewith a terminal disclaimer along with the appropriate fee. Thus, this rejection has been overcome and should be withdrawn.

Claims 87-96 have also been rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-29 of US Patent No. 5,773,217. In response, Applicant submits herewith a terminal disclaimer along with the appropriate fee. Thus, this rejection has been overcome and should be withdrawn.

Claims 87-96 have also been rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-6 of US Patent No. 6,753,457. In response, Applicant submits herewith a terminal disclaimer along with the appropriate fee. Thus, this rejection has been overcome and should be withdrawn.

Claims 87-96 have been provisionally rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-8 of copending Application No. 10/969,646. In response, Applicant submits herewith a terminal disclaimer along with the appropriate fee. Thus, this rejection has been overcome and should be withdrawn.

Claim Rejection–35 U.S.C. § 112, first paragraph

Claims 87-96 have been rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. According to the Examiner, "the specification does not reasonably provide enablement for any cell or stage of the cell cycle, where the nucleus, the cytoplasms and recipient eggs are of different species, and activation without incubation with the cytoplasm or development of an embryo." (Office Action at page 7). Thus, the Examiner concludes that the specification does not enable any person skilled in the art to make and use the invention commensurate in scope with the claims. (*See* Office Action at page 7). Applicant traverses.

As an initial matter, Applicant notes that claim 87 has been amended herein to specify that the recipient egg is from the same species as the somatic cell nuclei. Additionally, Applicant also notes that the specification provides detailed guidance for choosing suitable egg and CSF extracts. (*See* Specification at page 32, line 5 through page 34, line 12). For example, as noted in the specification, hardened Xenopus eggs are a good source for preparing an activating egg extract. (*See* specification at page 25, lines 33-35). Moreover, the specification also indicates that CSF extracts can also be obtained from non-induced Xenopus eggs. (*See* specification at page 35, lines 23-27). Likewise, numerous publications demonstrate the ability of Xenopus to reprogram human cells and sperm (Patent 5,480,772 (Appendix G), Neuber et al Biol of Reprod. 61:912-920 (Appendix H), and additional data has demonstrated the ability of Xenopus to reprogram pig, rat, and chicken sperm. (see specification page 14, lines 8-17). Moreover, Applicant notes that cross-species lysates/extracts (*e.g.*, rabbit reticulocytes lysates for in vitro translation, and HeLa cell extracts for gel shift assays.) have been used successfully as regents in research for many years demonstrating the conservation of proteins between species. Therefore, contrary to the Examiner's contention, Applicants submit that the ordinarily skilled artisan would be able to practice the method of claim 87 in order to reprogram a somatic cell nucleus in order to direct development of an embryo without undue experimentation.

Applicant has made a significant contribution to the field of whole animal cloning by discovering the critical element of the reprogramming process. Those skilled in the art will recognize that the art of nuclear transplantation for the purpose of cloning was already well-developed at the time of the instant invention (*see, e.g.*, specification, page 19, lines 5-11, describing the cloning of tadpoles). However, prior art difficulties encountered in the cloning of animals were the result of reprogramming somatic cell nuclei. The solution to these difficulties was discovered by the inventor of the instant invention. Specifically, the inventor discovered that contacting a somatic cell nucleus with a cystostatic factor cytoplasm (*i.e.* an MII oocyte cytoplasm) followed by an activating egg cytoplasm (*i.e.* an oocyte just prior to S-phase) reprograms the nucleus to allow development of a cloned animal (*i.e.*, by eliminating the somatic cell patterns of gene structure and function such as methylation patterns). (see specification page 47, lines 4-6 and pages 28-29, lines 35-36 and 1-9). The fundamental and unique properties of MII cytoplasm were the discovery that provided the solution to earlier problems associated with the cloning of whole animals.

This pretreatment phase is required to alter the chromatin structure and nuclear morphology of quiescent somatic cell nuclei. Subsequent treatment in activating cytoplasm is ultimately required to allow the chromatin ultrastructure to progress through MII and thus undergo DNA replication and formation of metaphase chromosomes without altering the integrity of the genetic information available for normal development.

The specification contains significant description of methods and experiments necessary to practice the claimed invention and establish the key parameters/incubation times to bring about nuclear reprogramming in vitro before being employed for whole animal cloning (see specification page 14 lines 18-28, Wangh et al J. of Cell Science 108: 2187-2196 (Appendix A), Ebert & Wangh Interdisciplinary Environmental Review 1 pg 9 para 3 (Appendix B)). As required by 35 USC 112 1st paragraph, Applicant contends that the specification sets forth the preferred embodiment of the invention (use of extracts) which is not meant to limited the scope of the invention but to reduce the inefficiency in the art. This statement is supported by the recent publication by Sullivan et al. Biol. of Reprod. 70:146-153 demonstrating the more efficient production of cows using a cystostatic factor contain extract. (Appendix C)

The claimed methods have been successfully employed in the somatic cell cloning of numerous species including mouse, rat, cat, dog, horse, rabbit, goat, cow, and pig. For example,

the concept of this invention has recently been utilized in the cloning of pigs. (*See* Polejaeva, et al., Nature 407:86-90 (2000) (Appendix D); Betthauser, et al., Nature Biotechnology 18:1055-59 (2000) (Appendix E)). These studies reported the successful production of cloned pigs using either a dual nuclear transfer approach or using *in vitro* maturation to MII stage oocytes followed by NT, then activation. Specifically, Polejaeva et al. used the dual nuclear transfer approach by first exposing nuclei to non-activating oocyte cytoplasm and subsequently retransferring the nuclei to an activated oocyte (an embryo in mitotic phase) for the generation of cloned pigs from quiescent somatic cell nuclei. On the other hand, Betthauser et al., used *in vitro* maturation of the oocyte to achieve MII oocytes (mitotic phase) followed by nuclear transfer. In addition, Campbell and coworkers (Nature 380: 64-66 (1996) (Appendix F)) addressed the importance of the different stages of oocyte development and its implication to appropriately target the proper cytoplasmic environment required for "reprogramming", thereby ultimately allowing a somatic cell nucleus to be totipotent for cloning.

Independent claims 87, 89, and 91 have been amended herein to specify that the somatic cell nucleus is activated by contacting the nucleus with the cytoplasm of an MII oocyte. The Examiner has already acknowledged that the claims are enabled for MII oocyte cytoplasm. (*See* Office Action at page 9).

The nucleus and the nuclear membrane is not a static environment but one that allows transport of necessary molecules/proteins (e.g., steroid hormone receptors, nuclear factors that have been translated in the cytoplasm) and would allow entry of the key factors necessary for reprogramming of the somatic cell nucleus. Furthermore, the preferred embodiment of the presently claimed invention specifically states that the efficiency of nuclear reprogramming can be enhanced by the pretreatment of the nuclei with mild detergent and protease to facilitate entry of key molecules into the nucleus. (see specification page 4, lines 33-37).

The Examiner has indicated that incubation of the nucleus in the presence of activating egg cytoplasm is critical to the activation method of this invention. According to the Examiner all such critical steps must be included for the claims to be enabled. (*See* Office Action at page 8). Applicant has herein amended independent claims 87, 89, and 91 to specify that the somatic cell nucleus is also contacted with an activating egg cytoplasm. Thus, Applicant submits that these claims, as amended herein, recite all critical steps.

Thus, for all of these reasons, Applicant contends that claims 87-96, as amended herein, are fully enabled by the as-filed specification. As such, this rejection should be withdrawn.

The Examiner has also rejected claim 97 under 35 U.S.C. § 112, first paragraph for lack of enablement. According to the Examiner, "at the time of filing the art recognized that nuclear transfer or cloning to produce a term animal was unpredictable. Even if the applicant's method results in a reprogrammed somatic cell nucleus, it is documented in the arena of nuclear transfer/cloning that a pregnancy does not necessarily mean live births." (Office Action at page 9). Applicant traverses.

Applicant submits that the disclosed invention has been successfully demonstrated to reprogram somatic cell nuclei for the production of whole or substantially complete cloned animals. To date, many of the problems associated with maintaining pregnancy have not been conclusively associated with inefficient nuclear reprogramming. Rather, the problems are likely to be inherent in the reproduction of higher organisms (The Scientist, April 25, 2005 v19(8) p13 1st paragraph "Normal development is an inefficient process-only 31 of every 100 human conceptions complete the journey" (Appendix I)) and the mechanical manipulation of oocytes/embryos often contribute to the failure to maintain pregnancy.

Nevertheless, Applicant submits that the instant specification provides the key steps required for the production of a cloned organism or a substantially whole organism. (*See, e.g.*, specification, page 59, lines 10-15). Moreover, several additional publications have reported the successful cloning of pigs and sheep by nuclear transfer. Specifically, Polejaeva et al., cloned pigs produced by double nuclear transfer from adult somatic cells; Betthauser et al., demonstrated the production of cloned pigs from *in vitro* systems using similar maturation oocyte programming *in situ*; and Campbell et al., cloned sheep by nuclear transfer from a cultured cell line. As noted in Betthauser et al., "We have systematically optimized each step in the NT procedure, including the source of oocytes and their maturation *in vitro*, the culture of the donor cells, the activation of oocytes following NT, and the *in vitro* culture of embryos and their transfer to recipients." (Betthauser et al., at page 1055).

To support this enablement rejection, the Examiner refers to the lack of success in cloning rabbits. However, Applicant submits that this lack of success does not necessarily mean

that the problems associated with cloning rabbits lies with the reprogramming of the somatic cell nucleus. In fact, as supported by Chesne et al. "Cloned rabbits produced by nuclear transfer from somatic cells" Nature 20:366-369 2002 (Appendix J), the problem associated with cloning rabbits was an asynchrony between embryo and recipient. However, cloned rabbits have now been successfully produced by this group of researchers using a rabbit oocyte at MII stage (i.e., using the claimed invention).

Moreover, in contrast to the Examiner's contention, the claimed methods do not denature the chromatin. Rather, the presently claimed methods decondense the chromatin. (*See, e.g.*, Specification at page 5, lines 29-32). Those skilled in the art recognize the need for reduced DNA damage in order for successful cloning, and the presently claimed methods provide ample guidance regarding how to accomplish reprogramming without damaging genetic material. (see specification page 6, lines 28-31).

Thus, Applicant submits that the invention accurately describes methods of effectively producing cloned animals through the appropriate maturation program for mammalian oocyte development, such that the oocyte will be at a developmental state for successful cloning applications. More specifically, the invention outlines the conditioning of the donor nuclei in non-activating cytoplasm and an activating cytoplasm. Therefore, Applicant contends that the invention is universally useful for cloning procedures. As such, anyone skilled in the art of animal embryology would be able to follow the teachings of the instant invention in order to successfully produce cloned animals.

The Examiner has also indicated that claim 97 is not enabled for its current breadth because, as of the filing date, cross-species nuclear transfer was not enabled. (*See* Office Action at page 10). In response, Applicant has amended claim 97 to specify that the somatic cell nucleus and the recipient egg are from the same species, which Examiner indicated is commensurate with the scope of the teachings provided by the specification. (Office Action at page 7).

Finally, the Examiner further notes that claim 97 "also lacks enablement as the only means known in the art to produce a new organism is by transferring the nuclear transfer embryo into the uterus of a female. An animal will not develop otherwise." (Office Action at page 11).

Applicant agrees that conditions for directing development of a new organism from an embryo include transferring the embryo (in this case, the nuclear transfer embryo) to a uterus of a female host (see specification page 58, lines 23-24), and this procedure was a well known and accepted step by skilled practitioners in the art of cloning at the time of the invention. As such, the step need not be recited by the claim. A patent need not teach, and preferably omits, what is well known in the art. (see *In re Buchner*, 929 F.2d 660, 661, 18 USPQ 481, 489 (Fed. Cir. 1991)). For example, as early as 1983, studies conducted on nuclear transplantation and subsequent development involved transfer to the uteri of pseudopregnant host females for further development. (McGrath and Solter, Science 20:1300, 1301 (1983) (Appendix K)). Laboratory manuals from 1986 state that embryos from the one-cell stage through to the blastocyte state are transferred into the reproductive tract of a pseudopregnant recipient to complete their development. (Manipulating the Mouse Embryo, a laboratory manual. Brigid Hogan, Frank Constantini, and Elizabeth Lacy, Cold Spring Harbor Laboratory 1986 (Appendix L)). Reference materials that teach cloning, such as Manipulating the Mouse Embryo, more specifically with oocyte/embryo collection, teach transferring the nuclear embryo separately from microinjection, NT, and ES cells because it is generic to all the techniques of cloning. To a person of ordinary skill in the art, transferring the nuclear transfer embryo to a host for further development was a well-known procedure at the time of filing and would not require undue experimentation. Therefore, Applicant believes the claim meets statutory requirements for enablement.

Thus, this rejection of claim 97 should be withdrawn.

Claim Rejection--35 U.S.C. § 112, second paragraph

Claim 97 has been rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Specifically, the Examiner has indicated that "[c]laim 97 is to a cloning a nonhuman animal, but the body of the claims state 'organism.' This is broader in scope than animal." (Office Action at page 12).

In response, Applicant has herein amended the body of claim 97 to recite "non-human animal", as is currently recited in the preamble of the claim. Thus, Applicant submits that this rejection has been overcome and should be withdrawn.

CONCLUSION

If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,

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Efficient reactivation of *Xenopus* erythrocyte nuclei in *Xenopus* egg extracts

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SUMMARY

Rapid genome replication is one of the hallmarks of the frog embryonic cell cycle. We report here that complete reactivation of quiescent somatic cell nuclei in *Xenopus* egg extracts depends on prior restructuring of the nuclear substrate and prior preparation of cytoplasmic extract with the highest capacity to initiate and sustain DNA synthesis. Nuclei from mature erythrocytes swell, replicate their DNA efficiently, and enter mitosis in frozen/thawed extracts prepared from activated *Xenopus* eggs, provided the nuclei are first treated with trypsin, heparin, and an extract prepared from unactivated, meiotically arrested, eggs. Optimal replicating extracts are prepared from large batches of unfertilized eggs that are synchronously activated into the cell cycle for 28 minutes (at 20°C). Because the *Xenopus* cell cycle progresses so rapidly, extracts prepared just a few minutes before or after this time have substantially lower DNA synthetic capacities. At the optimal time and temperature, eggs have just reached the G_1/S boundary of the first cell cycle. This fact was revealed by injecting and replicating an SV40 plasmid in intact unfertilized eggs as described previously. We estimate that under optimal conditions approximately 6.14×10^9 base pairs of DNA/per nucleus are synthesized in 30-40 minutes, a rate that rivals that observed in the zygotic nucleus.

The findings reported here are one step in our long term effort to develop a new in vitro/in vivo approach to nuclear transplantation. Nuclear transplantation in amphibian embryos has been used to establish that the genomes of many types of differentiated somatic cells are pluripotent. But very few such nuclei have ever developed into advanced tadpoles or adult frogs, probably because somatic nuclei injected directly into activated eggs fail to reactivate quickly enough to avoid being damaged during first mitosis. We have already shown that unfertilized eggs can be injected prior to activation of the first cell cycle. Future experiments will reveal whether in vitro reactivated somatic cell nuclei transplanted into such eggs reliably reach advanced stages of development.

Key words: *Xenopus* egg extract, in vitro nuclear reactivation, quiescent cell nuclei, erythrocyte, red blood cell, DNA synthesis in isolated nuclei, heparin treatment of nuclei

INTRODUCTION

Nuclear transplantation experiments are a corner stone of developmental biology and have proven beyond question that genomes of many types of differentiated somatic cells are pluripotent in so far as they can direct the development of embryos and larvae containing many types of differentiated cells. But in only one instance (Gurdon, 1962) have transplanted nuclei, those of larval tadpole intestinal epithelium, been shown to be totipotent. Moreover, no nucleus from a post-metamorphic somatic cell has yet generated a post-metamorphic animal. Historically the reasons given for these limitations are primarily technical (reviewed by Gurdon, 1986). It is argued that transplantation itself results in genetic damage because most somatic cell nuclei are unable to reenter the cell cycle quickly enough to replicate their entire genomes in the very short cell cycle of the egg. This hypothesis is supported by cytological observations that demonstrate that among transplant embryos those that are more advanced have more normal karyotypes. In addition, the nuclei of partial blastulae are often found upon second transfer to be more developmentally competent than the nuclei of perfect first transfer blastulae. Transplanted nuclei are therefore thought to escape major genetic damage if they skip karyokinesis at the first mitosis.

It follows from these observations that if somatic cell nuclei were able to enter the cell cycle more efficiently they would give rise to advanced tadpoles and even adult frogs more often. In order to test this prediction we are attempting to construct a new approach to nuclear transplantation in which nuclei are reactivated in vitro and are then transplanted into eggs at specific points in the cell cycle. We hope in this way to guarantee that a larger percentage of transplanted nuclei have a chance to direct development to their full potential.

As a first step toward establishing such a system, we have described a unique method for preparing unfertilized *Xenopus* eggs that allows them to be injected either before or after activation of the cell cycle (Wangh, 1989). We have also shown

that plasmid DNA replicates efficiently in activated eggs if it is first injected and incubated in the unactivated egg (Sanchez et al., 1992).

As a second step toward building a new nuclear transplantation system we have examined in vitro conditions leading to efficient reactivation and replication of whole frog erythrocyte nuclei. Nucleated erythrocytes were chosen because they are highly uniform, readily available terminally differentiated cells that resemble mature sperm in many ways. For instance, both types of cells are generated via a series of determinative divisions during which much of their cytoplasm and its organelles is discarded. At the same time nuclei in both types of cells become genetically inactive and pycnotic through the accumulation of special histones or protamines. In neither case, however, are the resulting quiescent genomes degraded, as is the case in apoptotic cells (Oberhammer et al., 1993).

In fact, as demonstrated by cell fusion (Harris, 1970) and nuclear transplantation experiments (Di Berardino and Hoffner, 1983) erythrocyte genomes are clearly intact and pluripotent. Immature frog erythroblast nuclei reactivate and support early development when injected directly into activated frog eggs (Brun, 1978). Even more remarkably, whole tadpoles develop when nuclei of fully differentiated erythrocytes are injected into maturing frog oocytes which are then induced to enter the cell cycle (Orr et al., 1986; Di Berardino et al., 1986; Di Berardino and Orr, 1992).

In our first report on in vitro replication of erythrocyte nuclei we showed (Coppock et al., 1989) that isolated nuclei from mature erythrocytes can swell and replicate in cytoplasmic extracts prepared from activated eggs, provided they are first subjected to mild trypsin digestion. We suggested that trypsin treatment frees the nucleus from constraints imposed by its surrounding cytoskeleton and its internal matrix. Trypsin treatment, however, has its limitations. For instance, the extent of replication was found to depend on the extent of trypsin digestion and levels of the enzyme required for very rapid replication tend to lyse nuclei causing them to clump (Leno and Laskey, 1991; L. J. Wangh, unpublished observations). In addition, replication of trypsin treated nuclei was not efficient in that complete genome replication took many hours. Finally, we observed that different preparations of egg extracts varied in their ability to support nuclear reactivation.

These experimental difficulties have now been overcome. Nuclei pretreated with low levels of trypsin are able to replicate completely and in a short period of time, provided they are further pretreated with heparin and with an extract prepared from mitotically arrested eggs before being placed in an interphase extract. Furthermore, batch-to-batch variations in cycling extracts have been reduced by carefully defining the point in the cell cycle at which extracts are prepared and by developing procedures for freezing and thawing extracts that do not inhibit synthesis of proteins need for DNA synthesis. Finally, our experiments illustrate that the nuclear/cytoplasmic ratio governs the extent of DNA synthesis and hence cell cycle progression in vitro. We conclude that a reliable and broadly applicable system is now available for studying reactivation and replication of quiescent cell nuclei. We discuss how in vitro nuclear reactivation can be combined with nuclear transplantation in the future.

MATERIALS AND METHODS

Preparation of *Xenopus* erythrocytes, isolation of their nuclei, pretreatment of nuclei with trypsin and heparin

Xenopus erythrocytes were obtained from large female animals as described by Coppock et al. (1989) with minor modification. The solutions used to wash cells contained the following mixture of protease inhibitors: 0.1 mM TPCK (*L*-1-tosylamide-2-phenyl-ethyl chloromtehyl ketone), 0.1 mM TLCK (*N*-α-*p*-tosyl-*L*-lysine chloromethyl ketone), 0.05 mM PMSF (phenylmethylsulfonyl fluoride), 5 μg/ml leupeptin, and 1 mg/ml SBTI (soybean trypsin inhibitor).

Erythrocyte nuclei were isolated by the lysolecithin/trypsin method of Coppock et al. (1989) with minor modification. The final concentration of lysolecithin was 40 μg/ml and the final concentration of Type XIII (Sigma) trypsin was 0.4 μg/ml from a stock of enzyme containing 11,700 units/mg. In some experiments after nuclei were treated with trypsin and washed they were suspended in 50 μg/ml heparin (Sigma Grade II, porcine, 192 units/mg) in NIB buffer and were incubated at 4°C for 60 minutes.

Preparation of egg extracts

All eggs for extract preparation were obtained, dejellied, and 'hardened' as described by Wangh (1989). Typically eggs from 10-15 animals are collected over a period of 4-6 hours and are then sorted and pooled to yield 10-20 ml of uniformly unactivated eggs. This approach minimizes frog-to-frog variations and provides sufficient quantities of frozen extract to last for several experiments.

Low speed cytostatic factor extract (CSF_L-extract)

CSF_L-extract was prepared from unactivated eggs that are arrested in meiotic metaphase II due to the presence of cytostatic factor. These eggs were rinsed at room temperature in EB buffer (50 mM K^+ gluconate, 250 mM sucrose, 1.5 mM $MgCl_2$, 10 mM Hepes, pH 7.5 with KOH), followed by rinsing them in EB buffer containing 5 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid), pH 7.5 with KOH. Eggs were then packed into polyallomer tubes previously treated with diethylpyrocarbonate. Each tube of eggs was aspirated as dry as possible and was then mixed with Versilube F-50 Oil (General Electric) (0.2 ml/ml eggs) prior to a gentle packing spin at room temperature as described by Murray and Kirschner (1989). Packed cells were then transferred to a cold (2°C) Sorvall HB4 centrifuge rotor and spun at 9,000 *g* for 15 minutes. The entire cytoplasmic supernatant between the yolk pellet and the lipid pellicle was collected into a syringe by puncturing the tube just above the black pigment layer. The viscosity of this extract was reduced by thorough mixing with 50 μg/ml cytochalasin B and the extract was supplemented with 1 mM EGTA/KOH, pH 7.5, and was then centrifuged again at 9,000 *g* for 15 minutes. The second cytoplasmic extract was collected and was either used fresh or was supplemented with 7.5% (v/v) glycerol and was frozen by spotting 20 μl droplets onto a block of aluminum immersed in liquid nitrogen.

High speed cytostatic factor extract (CSF_H-extract)

CSF_H-extract was prepared as described above except the second cytoplasmic supernatant was collected after centrifugation for 2 hours at 35,000 rpm in a Beckman SW50.1 rotor. Under these conditions membrane vesicles in the extract form distinct layers within the cytoplasm, but they do not pellet. All layers above the hard pellet at the bottom of the tube were collected along with the clear cytoplasm above them.

ACT-extract

ACT-extract was prepared from activated eggs that were first collected, washed, sorted, and pooled as described above. The eggs were then washed three times with activation buffer (4 mM NaCl, 0.7

mM potassium gluconate, 10 mM Hepes, 2 mM $MgCl_2$, 0.6 mM $Ca(NO_3)_2$, pH 7.4) and were activated for 10 minutes by addition of calcium ionophore A23187 to 100 nM. During this step all eggs in the population should exhibit distinct animal pole capping. The eggs were then rinsed in 1.5× NKH supplemented with 2 mM $MgCl_2$, 0.6 mM $Ca(NO_3)_2$ (1× NKH is 40 mM NaCl, 2.5 mM KCl, 7.5 mM Hepes, pH 7.4) and were incubated for a further period of 0-30 minutes. After the desired total time the eggs were thoroughly washed in ice-cold EB buffer and were then processed in the cold as described above for low speed CSF-extract, except that potassium EGTA was not added to the first cytoplasmic supernatant.

Incubation of nuclei in CSF- and ACT-extracts

For each experiment the required amount of CSF- and/or ACT-extract was thawed on ice and was supplemented with creatine phosphate to 0.4 mM and creatine phosphokinase to 0.4 μg/ml in a total volume not greater than 1/10th volume of the extract. Both the low speed and high speed CSF-extracts were further supplemented with recrystallized β-glycerol-PO_4 to 80 mM and were also supplemented with $CaCl_2$ to a final concentration of 0.1 mM. Erythrocyte nuclei prepared via the lysolecithin and trypsin pretreatment protocol described above were added to low speed or high speed CSF-extracts at a final concentration of 2,000 nuclei/μl, or directly to ACT-extract at a final concentration of 200 nuclei/μl. Alternatively, the CSF-extract containing nuclei was diluted 1:10 in ACT-extract.

Before being warmed to 23°C each ACT-extract containing nuclei was subdivided into aliquots and supplemented according to the design of the particular experiment underway. For analysis of rate of DNA synthesis extracts received 0.2 μCi/μl of high specific activity [α-^{32}P]dCTP (New England Nuclear). For analysis of the extent of replication extracts were supplemented with 300 μM BrdUTP (5-bromo-2′-deoxyridine 5′ triphosphate) and 300 μM $MgCl_2$. For analysis of DNA replication by fluorescence microscopy extracts were supplemented with 16 μM biotinylated-11-dUTP (Enzo Diagnostics) and 16 μM $MgCl_2$. In some cases extracts were also treated with aphidicolin to 50 μg/ml in 0.2% DMSO (dimethyl sulfoxide), an amount sufficient to block DNA replication completely, or 100 μg/ml cycloheximide, an amount sufficient to block protein synthesis completely.

Sample collection and analysis

The above reaction mixture was incubated at 23°C and was sampled and analyzed as follows:

[^{32}P]dCTP-labelled DNA and agarose gel analysis

During the experiment, 5 μl samples were frozen on dry ice. Each samples was then thawed and mixed with 7 μl of a sample buffer containing 80 mM Tris-HCl, pH 8.0, 8 mM EDTA (ethylene diamine tetraacetic acid), 0.13% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% Bromophenol Blue and 1.7 mg/ml proteinase K (Boehringer Mannheim). After incubation at room temperature for at least 2 hours samples were loaded onto a 0.8% agarose gel prepared in TAE buffer (40 mM Tris-HCl, 5 mM Na^+ acetate, 1 mM EDTA, pH 8.0 with glacial acetic acid) and were run overnight at 50 V. This procedure separates incorporated and unincorporated label. The gel containing the incorporated counts was washed repeatedly in water and was then dried and scanned with a Molecular Dynamics PhosphorImager.

BrdUTP-labelled DNA and CsCl density gradient analysis

During the experiment, 5 μl samples were frozen on dry ice. Specific samples were then thawed and digested in 445 μl of a proteinase K solution consisting of 10 mM EDTA, 20 mM Tris-HCl, pH 8, 0.5% sarcosyl, 300 μg/ml Proteinase K for 1 hour at 60°C. The sample was then adjusted to 200 mM NaCl in a final volume of 500 ml and was sheared by passing through a 25 G needle 10 times and a 27 G needle 5 times. The DNA was fractionated by diluting it in 3.5 ml CsCl in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), final refractive index of 1.4030, followed by centrifuging it for 20 hours at 20°C in an SW50.1 rotor. The resulting gradient was fractionated from the bottom into 30-40 100 μl fractions. Those fractions containing refractive indices in the light-light to heavy-heavy range were dot blotted and probed with ^{32}P-labelled total *Xenopus* DNA (Brown et al., 1983).

Biotinylated-dUTP labelled DNA and Texas Red/streptavidin staining

During the experiment, 5 μl samples were mixed with 100 μl of freshly prepared EGS fixative and incubated at 37°C for 30 minutes. Fresh EGS fixative was prepared by diluting a 100 mM stock of ethylene glycol-bis(succinic acid *N*-hydroxysuccinimide ester; (Sigma) in DMSO 1:100 into NIB, our standard nuclear isolation buffer (Coppock et al., 1989). For staining, 50 μl fixed nuclei were mixed with 75 μl of TR stain and were incubated 90 minutes at room temperature. (TR stain is 680 μl 3× NKH and 100 μl 4% BSA (bovine serum albumin fraction V) and 7 μl Nonidet P40 and 20 μl Texas Red/streptavidin (Gibco/BRL).) Each sample was then centrifuged onto a coverslip through a cushion of 30% glycerol and was then counter stained with Hoechst 33258 stain at 2 μg/ml.

FITC anti-lamin staining

EGS fixed nuclei prepared as described above were stained overnight with monoclonal antilamin antibody L6 847 (gift of Dr R. Stick; Stick and Hausen, 1985) followed by counter staining with FITC goat anti-mouse IgG (Kirkegaard and Perry, Lab. Inc.).

Measurements of histone H1 kinase activity

During the experiment, 5 μl samples were frozen on dry ice. Each sample was then thawed and immediately mixed with 45 μl of H1K buffer (H1K buffer is 80 mM recrystallized β-glycerol-PO_4, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT (dithiothreitol), 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.3, with KOH.) A 2 μl sample was then added to an additional 10 μl H1K buffer and 8 μl of H1K cocktail and the reaction was incubated for 15 minutes at room temperature. (H1K cocktail is 0.4 mg/ml histone H1 (Sigma), 10 μM cAMP-Dependent Protein Kinase Inhibitor (Peninsula), 100 μM ATP, and [^{32}P]ATP at 4 μCi/100 μl cocktail.) At the end of the reaction each sample was spotted to Whatman P81 paper and was rinsed extensively in 1% phosphoric acid and then 95% ethanol and was then dried and analyzed using a Molecular Dynamics PhosphorImager.

Measurements of DNA replication intermediates in whole eggs via plasmid injection

Unactivated eggs were toughened and injected as described by Wangh (1989) and Sanchez et al. (1992). The plasmid was a 7.6 kb construct containing the SV40 origin of replication (kindly provided by M. Bradley). Plasmid DNA recovered from injected eggs was linearized with *Sma*I at 30°C, was mixed with 300 ng *Hin*cII digested φX174 DNA as carrier, and was run in a 0.4% agarose gel in 1× TEA for 22 hours at 41 V. The region of the gel containing DNA in the 6-20 kb range was acid nicked for 15 minutes and was blotted to a Zetaprobe membrane (Bio-Rad). Replication intermediates were detected by hybridization to the same plasmid ^{32}P-labelled by the random primer technique. These conditions allow the DNA molecules to separate as a function of their mass. As a result, the replication intermediates, which are larger than unit length linears, migrate at a slower rate and form a smear. These intermediates are not seen in experiments in which aphidicolin is added to the eggs to block DNA replication (results not shown). In addition, these intermediates are sensitive to *Dpn*I digestion, as expected for partially replicated molecules (Sanchez et al., 1992). Two-dimensional gel electrophoresis of plasmid replication intermediates was carried out as described by Brewer and Fangman (1987).

RESULTS

Trypsin treatment alone is not sufficient for complete nuclear reactivation

Our original report (Coppock et al., 1989) demonstrated that trypsin digestion of non-histone nuclear proteins is required for in vitro reactivation of mature erythrocyte nuclei. Trypsin-treated nuclei were incubated in either fresh or frozen/thawed extracts prepared from eggs activated for 10 minutes (ACT_{10}-extract). Nuclei handled in this way swell, acquire egg-type lamin, and initiate DNA synthesis in an extract prepared from activated *Xenopus* eggs, provided they are trypsin pretreated (Figs 1 and 2). Reactivation in this system, however, is inefficient. DNA synthesis only begins after a lag period of about 2 hours and then proceeds relatively slowly for several hours. In addition, some nuclei treated in this way appear to consist of a small region of compact chromatin and a large herniated region of decondensed chromatin. These observations indicated to us that trypsin pretreatment alone is not sufficient to precondition the erythrocyte nucleus for efficient reactivation and replication. As described below we sought to remedy this problem by further altering nuclear structure before initiating replication.

Combined trypsin pretreatment and incubation in CSF-extract prior to replication

Two lines of evidence suggested that incubation of trypsin-pretreated nuclei in extracts prepared from unactivated *Xenopus* eggs (CSF-extract) might further enhance their ability to reactivate and replicate when transferred in an extract prepared

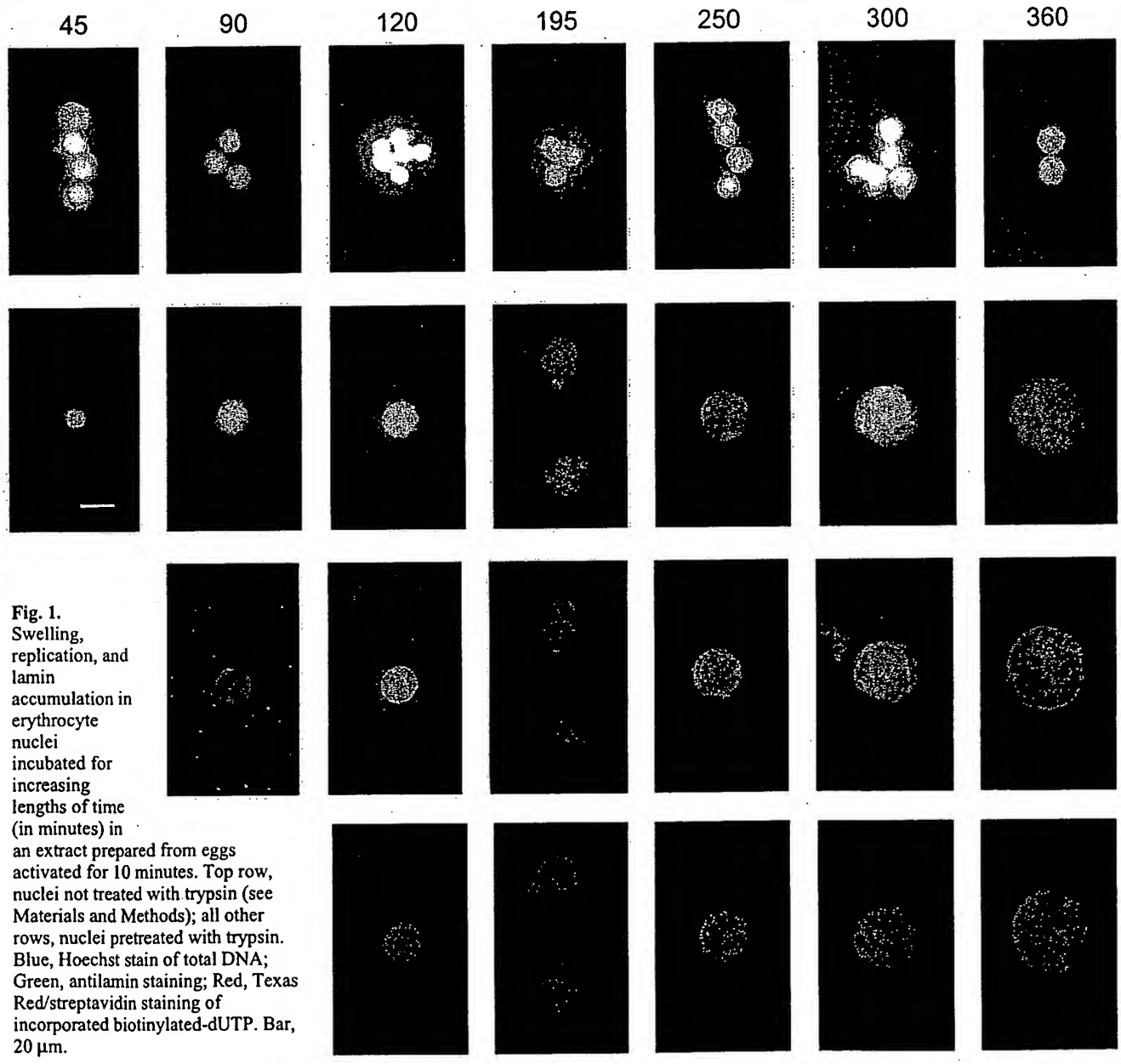

Fig. 1. Swelling, replication, and lamin accumulation in erythrocyte nuclei incubated for increasing lengths of time (in minutes) in an extract prepared from eggs activated for 10 minutes. Top row, nuclei not treated with trypsin (see Materials and Methods); all other rows, nuclei pretreated with trypsin. Blue, Hoechst stain of total DNA; Green, antilamin staining; Red, Texas Red/streptavidin staining of incorporated biotinylated-dUTP. Bar, 20 μm.

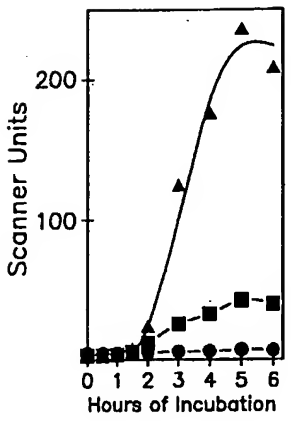

Fig. 2. [^{32}P]dCTP incorporation demonstrates quantitatively that trypsin-treated nuclei (▲), but not untreated nuclei (●), replicate in activated egg extract (see Materials and Methods). [^{32}P]dCTP-labelling also demonstrates that nuclei that have not been treated with trypsin but have been pre-incubated in a CSF_L-extract (■), are able to synthesize some DNA when transferred to an ACT-extract (see Materials and Methods).

from activated eggs. First, Di Berardino and her colleagues demonstrated that nuclei of erythrocytes injected into maturing oocytes go on to reactivate and develop to advanced stages (Orr et al., 1986; Di Berardino et al., 1986, Di Berardino and Orr, 1992). Second, we have shown that plasmid DNAs replicate efficiently in activated eggs, provided they are first injected and incubated in unactivated eggs (Wangh, 1989; Sanchez et al., 1992). As described below, this new pretreatment strategy eventually proved successful.

As illustrated in Fig. 3, incubation of trypsin-treated nuclei in low speed CSF_L-extract 30 minutes at 25°C led to subsequent rapid replication, while incubation in buffer alone or CSF_L-extract at 4°C did not. However, the effect of CSF_L-extract was not simple since incubation at 25°C for 120 minutes was less stimulatory than incubation for 30 minutes. Microscopic examination of the nuclei showed the reason why (Fig. 4). Trypsin-treated nuclei at the start of the reaction are spherical and are bounded by a discrete envelope. During the initial 30-60 minutes of incubation (depending on the potency of the extract) nuclei loose their envelopes and their chromatin becomes further compacted taking on a roughened appearance. These changes are undoubtedly due to the

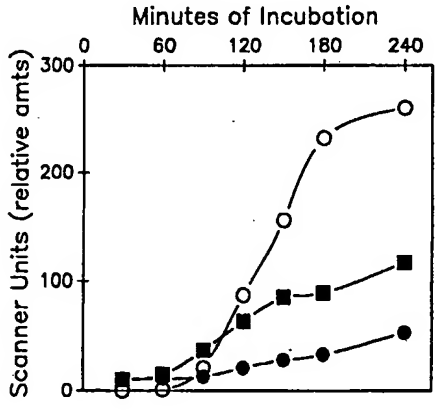

Fig. 3. DNA replication in trypsin-treated nuclei that were pre-incubated in CSF_L-extract for different lengths of time and temperatures and were then diluted to ACT-extract containing [^{32}P]dCTP. CSF pre-incubation times and temperatures: 30 minutes at 25°C (○); 120 minutes at 25°C (■); 120 minutes at 4°C (●).

presence of histone H1 kinase activity in the CSF-extract and do not occur if 6-dimethylamino purine, a kinase inhibitor, is added to the extract (Lee and L. J. Wangh, unpublished). As shown below, nuclei in this state proved ideally suited for subsequent chromatin decondensation, new envelope assembly, and replication once they are transferred to an ACT-extract. However, when incubation in CSF-extract is continued for 120 minutes, nuclei disperse into chromosome-like fragments and no longer serve as efficient substrates for nuclear assembly and replication. Subsequent experiments revealed that high speed CSF_H-extracts are more effective than low speed CSF_L-extracts in preconditioning nuclei. We therefore routinely use high speed extracts and shift the incubate from 25°C to 4°C after 30-60 minutes to prevent dispersion of chromosomes.

Preparation of the best ACT-extract and the timing of S-phase in the first cell cycle

Before we could proceed to a detailed examination of how pretreated nuclei replicate in ACT-extract, we had to establish which ACT-extract exhibits the highest DNA synthetic capacity. A priori it seemed likely that because activated eggs traverse the cell cycle so quickly, small differences in the

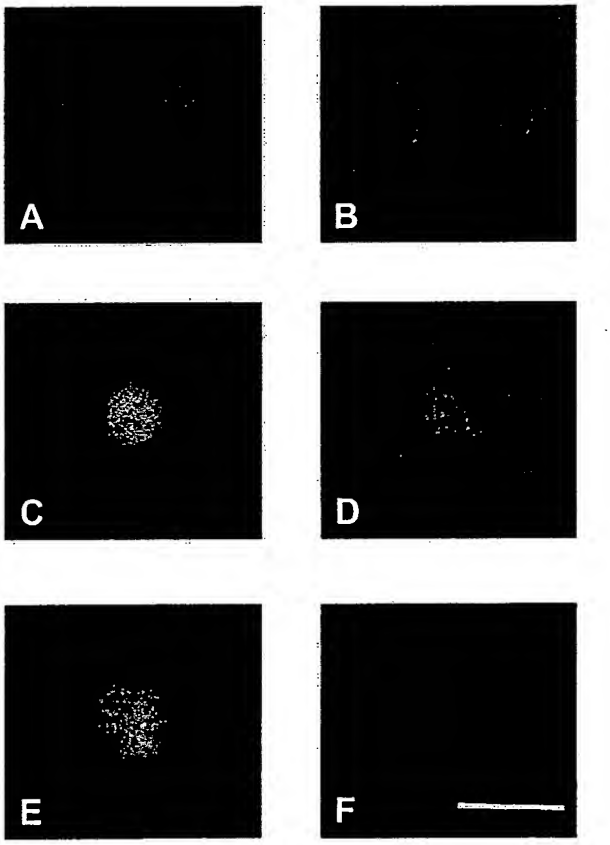

Fig. 4. Appearance of cell nuclei during pretreatment steps. (A and B) Untreated control nuclei, note presence of nuclear envelope. (C and D) Lysolecithin/trypsin treated nuclei, note presence of nuclear envelope. (E) Nuclei incubated in CSF_L-extract for 60 minutes at 25°C plus 60 minutes at 4°C, note absence of nuclear envelope or (F) 120 minutes at 25°C, chromosome-like fragment. Bar, 10 μm.

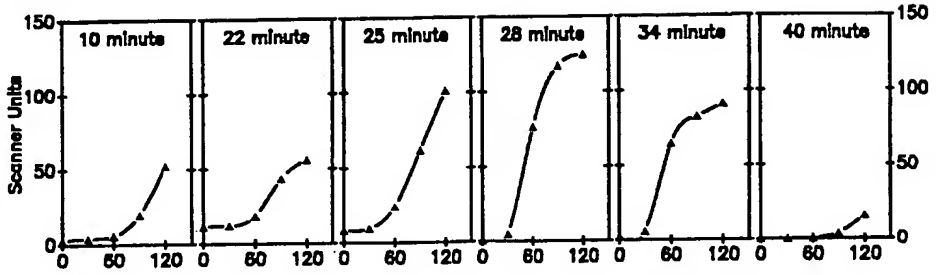

Fig. 5. [^{32}P]dCTP incorporation into the DNA of trypsin/CSF$_H$-extract pretreated-nuclei that were then incubated in different batches of frozen/thawed ACT-extract. These ACT-extracts were prepared from eggs that had been activated en masse with calcium ionophore for 10 minutes (see Materials and Methods) and were then allowed to proceed into the first cell cycle for increasing lengths of time before being crushed.

timing of extract preparation would have major effects on their intrinsic replicative capacity. In addition, differences in the synchrony of egg activation might cause batch-to-batch variations in extract quality (Coppock et al., 1989).

We have demonstrated previously that large numbers of *Xenopus* eggs can be amassed and synchronously activated by first toughening their vitelline envelopes (Wangh, 1989). We therefore used this technique to activate batches of eggs from which extracts were prepared and frozen at closely spaced intervals. All of these extracts were then tested for their ability to support replication in the same trypsin/CSF$_H$-pretreated nuclei. The results in Fig. 5 demonstrate that the DNA synthetic capacity of ACT-extracts first increases and then decreases as eggs proceed through the cell cycle. At 20°C the most active extract, ACT$_{28}$, is obtained from eggs that are washed and crushed in ice-cold EB-buffer 28 minutes after activation. Nuclei in ACT$_{28}$ begin replication sooner, replicate faster, and replicate more completely than in other extracts. Fig. 6 demonstrates that even in freeze/thawed ACT$_{28}$-extract efficient replication depends on ongoing protein synthesis prior to the start of replication. Addition of cycloheximide at t=0 decreases DNA synthesis while addition after 30 minutes, just at the start of S-phase, has little affect.

In light of these findings, we wanted to know what cell cycle event takes place in unfertilized eggs 28 minutes after activation to account for the high DNA synthetic capacity of extracts prepared at this time. In order to answer this question we turned to the technique of plasmid DNA injection which we have also described previously (Wangh, 1989; Sanchez et al., 1992). By buffering extracellular calcium levels to 1-2 μM free Ca^{2+}, plasmid DNAs can be injected into unactivated eggs without triggering the start of the cell cycle. After the prick site heals calcium ionophore treatment can then be used to activate these eggs and this leads to one or more rounds of plasmid DNA replication.

Several hundred eggs were therefore injected with a plasmid derived from SV40 virus and after 5 hours of incubation were synchronously activated. Groups of 3-5 eggs were then sampled at one minute intervals during the middle of the first cell cycle. The plasmid DNA was then recovered and linearized to establish when partially replicated molecules first appear. Fig. 7 shows that replication intermediates begin to accumulate about 27 minutes after activation and a high concentration of these intermediates is present between 34 and 57 minutes. Two-dimensional neutral-neutral agarose gel electrophoresis (not shown) confirmed that the slowly migrating molecules are indeed Cairn's type replication intermediates containing bubbles and double-Y structures (Brewer and Fangman, 1987). This experiment, together with our in vitro assay of nuclear replication above, suggests that right up until the start of S-phase the egg cytoplasm synthesizes and accumulates one or more proteins that promote nuclear reactivation.

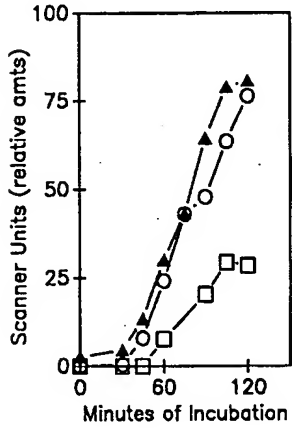

Fig. 6. Maximal DNA synthetic capacity of frozen/thawed 28 minutes ACT-extract depends on early protein synthesis. Control (▲): cycloheximide added at t=0 (□); cycloheximide added at t=30 (○).

Optimized conditions permit highly synchronous, complete, erythrocyte nuclear DNA replication

Now that we had adjusted both nuclear pretreatment and extract preparation, we were in a position to examine the entire time course of erythrocyte nuclear reactivation and replication. Accordingly, nuclei pretreated with trypsin and CSF$_H$-extract were then diluted into ACT$_{28}$-extract and subdivided into aliquots. One aliquot received [^{32}P]dCTP for measurement of replication kinetics, one received BrdUTP for measuring the extent of replication, one received biotinylated-dUTP for cytological detection of replication, and one aliquot was set aside for analysis of histone H1 kinase activity.

[^{32}P]dCTP-labelling revealed that under these conditions DNA synthesis began after a lag period of only 30 minutes, rose rapidly until 75 minutes, and then more slowly for an additional 75 minutes (Fig. 8A). By 30 minutes all nuclei had swelled slightly and had begun to incorporate biotinylated-dUTP. As S-phase continued the nuclei swelled to a very large size and then began to condense. The chromatin in these nuclei

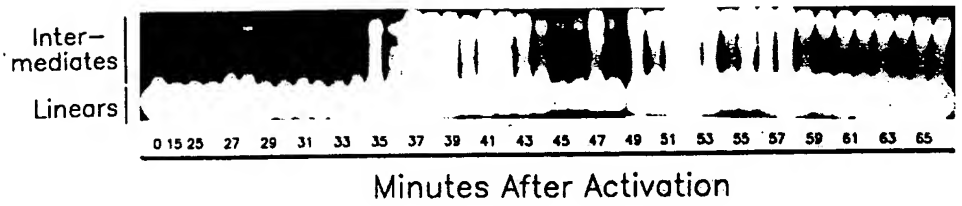

Fig. 7. SV40 plasmid DNA replication intermediates begin to accumulate about 27 minutes after activation and a high concentration of these intermediates is present between 34 and 57 minutes. Negative image of autoradiogragh.

pulled away from the nuclear envelope, but the nuclear envelopes did not break down. There was no measurable histone H1 kinase activity throughout this period of time. BrdUTP density labelling at 120 minutes revealed that a major fraction of the DNA had a heavy-light density, indicative of once-replicated DNA. However a significant fraction of the DNA still trailed back to the light-light position on the gradient, indicating that round-one replication was not yet complete (Fig. 8B).

These results demonstrate that, as compared to our original procedure (Figs 1 and 2), our new two-step pretreatment procedure (trypsin/CSF-extract) combined with ACT_{28}-extract had significantly shortened the lag time before the start of replication and had also improved the initial rate of replication. We were troubled, however, by the observation that nuclear replication proceeded in two phases, was still not complete, and failed to progress into mitosis.

The failure to observe mitosis could be due to the use of freeze/thawed extracts which might not synthesize B cyclins at sufficiently high rates to activate $p34^{cdc2}$ kinase (Murry and Kirschner, 1989). However, our extracts were able to generate histone H1 kinase activity when they were incubated without nuclei. This observation indicated to us that the nuclear substrate rather than the egg extract was at fault. The failure of nuclei to enter mitosis might be due to their failure to complete replication, since ongoing DNA synthesis as well as inhibition of ongoing DNA synthesis are known to block cell cycle progression into mitosis in *Xenopus* egg extracts (Dasso and Newport, 1990). Perhaps one or more proteins of quiescent erythrocyte nuclei, like histone $H1^0$ or MENT protein (Grigoryev and Woodcock, 1993), remains bound to chromatin and thereby prevents rapid and complete chromatin decondensation and replication. Reactivation of chicken erythrocyte nuclei in activated egg extracts has been shown by Blank et al. (1992) to involve gradual histone $H1^0$ phosphorylation and dissociation from chromatin, as well as the phosphorylation of several other histones. Based on this knowledge we sought to enhance erythrocyte nuclear reactivation still further by introducing yet another step into our pretreatment protocol.

Heparin pretreatment enhances round-one replication and thereby promotes mitosis

Heparin is a polyanion and has been used to swell rat liver nuclei (Kraemer and Coffey, 1970) and to enhance decondensation and replication of human sperm nuclei in *Xenopus* egg extracts (Montag et al., 1992). We hoped that heparin treatment of trypsin pretreated nuclei would increase both their rate and extent of replication after dilution into CSF- and ACT-extracts; Fig. 9 demonstrates that this was indeed the case. In addition only the reaction containing heparin-treated nuclei exhibited an abrupt increase in histone H1 kinase activity, a clear indication that replication was complete and the cytoplasm was free to advance into mitosis. Fluorescent microscopy (not shown) confirmed that heparin-treated nuclei swelled and incorporated biotinylated-dUTP very rapidly, started to condense their chromatin as soon as replication stopped (60 minutes), and underwent nuclear envelope breakdown coincident with the rise in histone H1 kinase activity (120 minutes). When histone H1 kinase activity began to fall at the end of mitosis (180 minutes) new nuclear envelopes formed.

We conclude that the combined three step pretreatment procedure (trypsin/heparin/ CSF-extract) followed by ACT_{28} extract had, at last, converted quiescent erythrocyte nuclei into decondensed structures similar to those of the early embryo. The maximum rate of DNA replication achieved in each fully reactivated erythrocyte nucleus, approximately 6.14×10^9 base pairs synthesized in 30-40 minutes, rivals that observed in the zygotic nucleus.

DISCUSSION

Rapid genome replication is one of the hallmarks of the frog embryonic cell cycle. Our results demonstrate that efficient and complete genome replication in vitro requires chromatin restructuring before replication actually starts, as well as use of an interphase extract which exhibits a high capacity for rapid and sustained DNA synthesis. Our final procedure for in vitro reactivation employs three chemically defined pretreatment steps followed by two incubations in extracts prepared from unactivated and activated *Xenopus* eggs. This experimental system is likely to prove useful for analysis of how many types of quiescent somatic cell nuclei reenter the cell cycle because it is both flexible and convenient, particularly because egg extracts are prepared in advance.

In the case of mature erythrocyte nuclei restructuring begins with gentle cell lysis with lysolecithin which leaves the cytoskeleton and nuclear matrix intact. These cell structures are degraded with trypsin at such low levels that histone $H1^0$ and core histones remain intact (Coppock et al., 1989). Heparin is employed next causing the nuclei to swell slightly. Although we have not yet examined the biochemistry of this step it is likely to involve extraction of histone $H1^0$. Blank et al. (1992) have analyzed biochemical changes taking place in chicken erythrocyte nuclei incubated in activated frog egg extracts. They observed that histones $H1^0$, H2A, and H4 become phosphorylated and $H1^0$ is eventually displaced as the nuclei gradually reactivate. It is likely that in our system these phosphorylation events, together with nuclear envelope breakdown, take place during incubation of nuclei in CSF-extract which has relatively high levels of histone H1 kinase activity. Nuclei incubated for extended periods in CSF-extract dissociate into chromosome-like structures demonstrating that they have undergone profound structural changes.

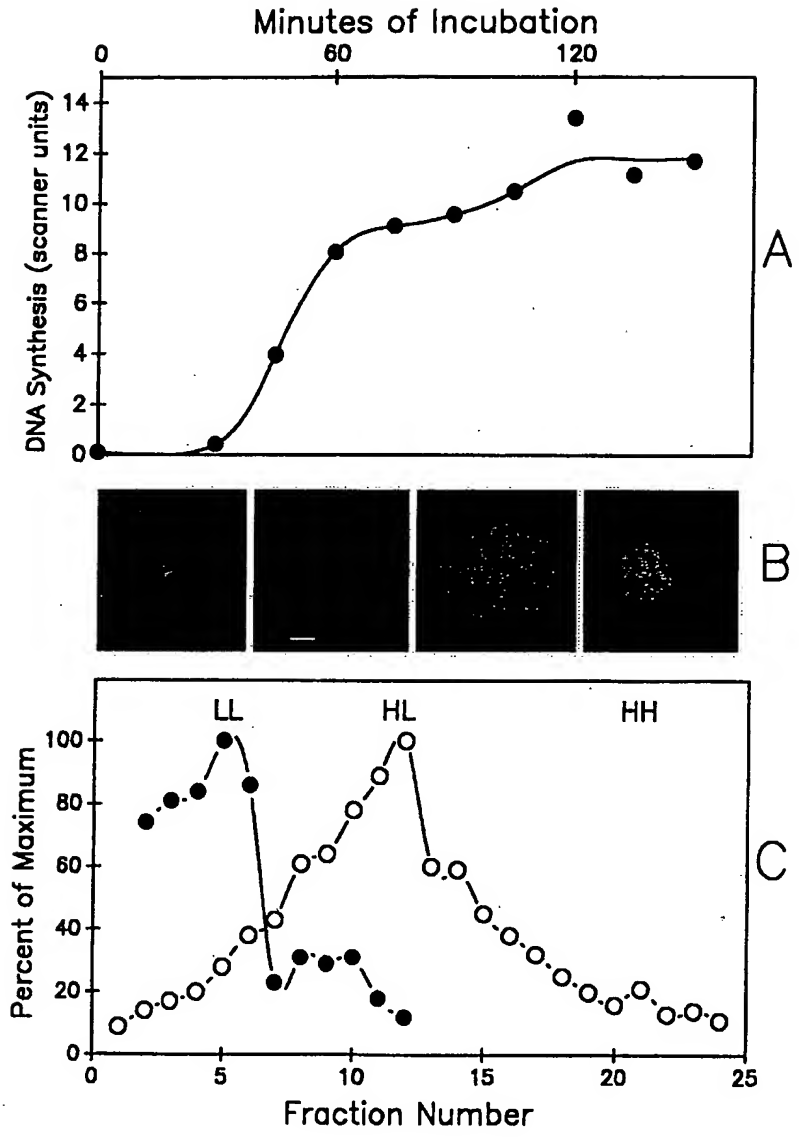

Fig. 8. Responses of trypsin/CSF_H-extract pretreated nuclei to ACT_{28}-extract. (A) DNA synthesis in [^{32}P]dCTP labelled nuclei. (B) Appearance of nuclei (from left to right): t=0 nucleus stained with Hoechst and photographed with a combination of fluorescent and bright field optics; t=30 nucleus stained with Texas Red/streptavidin for incorporated biotinylated-dUTP; t=120 nucleus stained with Hoechst; t=135 nucleus stained for lamin (green) and replicated DNA (Texas Red). Bar, 10 μm. (C) CsCl density gradient analysis of DNA recovered from nuclei incubated with BrdUTP for 0 minutes (●) and for 120 minutes (○).

Nuclear reactivation in our system is carried out by diluting the fully pretreated nuclei into interphase extracts prepared from chemically activated eggs that are crushed just as they reach the G_1/S boundary of the first cell cycle. These extracts contain the highest possible concentrations of all of the factors needed to initiate and complete DNA synthesis in the shortest possible time. This result implies that fully reactivated nuclei, like nuclei of the early embryo, may initiate DNA synthesis simultaneously in many closely spaced relatively small replicons. Reactivated nuclei go on to complete replication and first mitosis. This proves that ACT_{28}-extract is fully cell cycle competent, even though it has been frozen and thawed.

The experiments described here also illustrate the remarkable synchrony with which large numbers of *Xenopus* eggs traverse the first cell cycle, provided their vitelline envelopes are stabilized and the population is culled of prematurely and partially activated eggs (Wangh, 1989). As a byproduct of these investigation we have established for the first time that DNA synthesis begins in unfertilized *Xenopus* eggs 27 minutes after ionophore activation at 20°C and continues for approximately 30 minutes. This is very similar to the timing of pronuclear replication in fertilized eggs that begins 28-30 minutes after fertilization and is completed in the next 22-24 minutes (Gerhart, 1980). In our system almost fully replicated plasmid DNA molecules persist for at least ten minutes after the active portion of S-phase ends. This suggests that *Xenopus* eggs have a higher capacity for initiating the replication of circular plasmid molecules than for completing it. This observation is consistent with our previous finding that many nearly replicated plasmid molecules are degraded when *Xenopus* eggs enter mitosis (Sanchez et al., 1992).

Finally, the conditions that we have defined for erythrocyte nuclear replication in vitro resemble those used by Di Berardino and her colleagues to obtain advanced Rana tadpoles from the nuclei of mature erythrocytes. In both cases intact nuclei are gently lysed and are then exposed to meiotic

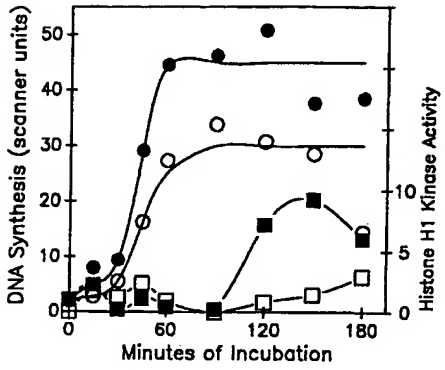

Fig. 9. DNA synthesis (●, plus heparin; ○, no heparin) and histone H1 kinase (■, plus heparin; □, no heparin) activity in reactions containing nuclei which had and had not been treated with heparin. Trypsin-treated nuclei were incubated in the presence or absence of 50 μg/ml heparin in NIB buffer for 60 minutes at 4°C and were then diluted into CSF_H-extract at 2,000/μl followed by 1:10 dilution into ACT_{28}-extract containing [^{32}P]dCTP (see Materials and Methods).

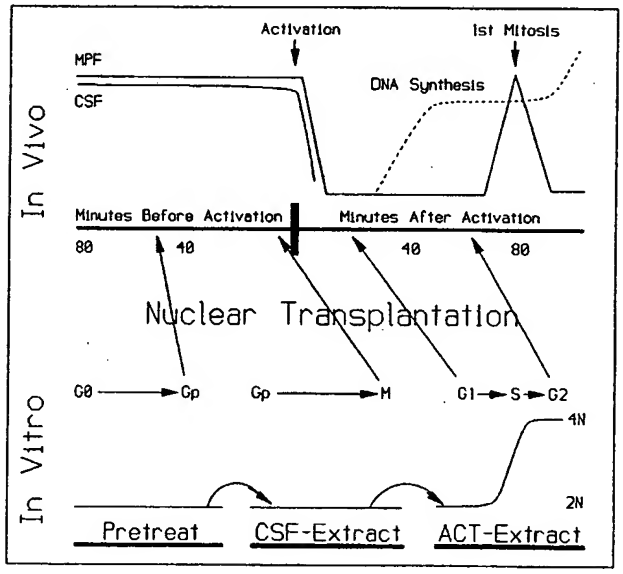

Fig. 10. A new in vitro/in vivo approach to nuclear transplanation. Quiescent (G_0) nuclei can be pretreated in vitro with trypsin and heparin to generate pretreated (Gp) nuclei. These nuclei can then either be incubated in CSF-extract (bottom row) or transplanted into unactivated eggs (vertical arrow). Similarly, CSF-treated nuclei that have become mitotic (M) can either be diluted into ACT-extract (bottom row) or transplanted into unactivated eggs (vertical arrow) which are then activated. Finally, nuclei reactivated in ACT-extract, either before (G_1) or after (G_2) they have undergone DNA synthesis (S) in vitro, can be transplanted into activated eggs (vertical arrows) either before or after first S-Phase in vivo. The eggs used in these experiments can be prepared in advance and held in the unactivated egg by stabilizing their vitelline envelopes. They then can be enucleated with UV-light and activated by calcium ionophore treatment at specific times either before or after receiving a transplanted nucleus.

metaphase egg cytoplasm before they enter the cell cycle. The combination of our in vitro replication system with our method for injecting eggs either before or after they are activated into the cell cycle (Wangh, 1989), suggests a new approach to nuclear transplantation (Fig. 10). Erythrocyte nuclei at any stage during reactivation and replication in vitro can now be injected into eggs at any point in the cell cycle. Future experiments will establish whether nuclei that have been preconditioned for efficient replication in vitro go on to divide and direct development. It is of course possible that we still have not succeeded in reactivating the red cell genome, even worse, that we have introduced new technical problems that artifactually compromise development. However, if the new in vitro/in vivo approach is successful, we should be able to rigorously establish the full developmental capacities of mature erythrocyte nuclei.

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GENETIC ENGINEERING VIA TRANSGENICS AND CLONING: PROSPECTS, VALUE AND ENVIRONMENTAL IMPACT

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&
Dr. Lawrence J. Wangh, Brandeis University, USA

ABSTRACT

Transgenic manipulation of living plants and animals is one of the new technologies that will be employed in the 21st Century to improve the efficiency of food production, as well as the synthesis of medically and socially valuable biological products. While the technologies for creating transgenic plants and animals are now in the early testing stage, it is already possible to imagine creation of seed plants that produce important proteins, new breeds of pigs and fish that grow faster, are leaner, and require less grain and goats or cows that produce human insulin for treating diabetes, or antibodies for fighting cancer. The list of such products is already very long and is expected to grow rapidly as the technology for producing transgenic plants and animals improves.

I. FORWARD

Environmental Impact of Transgenic Animals: Benefits and Risks. The number of people on earth is now 6 billion and will climb to at least 8 billion by 2050 despite declining birthrates in many countries, unless mortality rates increase significantly. Two inescapable facts accompany the shear magnitude of these numbers: first, human beings will place enormous burdens on the biological and physical resources of the earth for the foreseeable future. Second, environmental disaster will only be avoided in the coming decades via a combination of human understanding, ingenuity, and a commitment to global management. Science and technology, particularly biotechnology, will have a major role in addressing these global challenges.

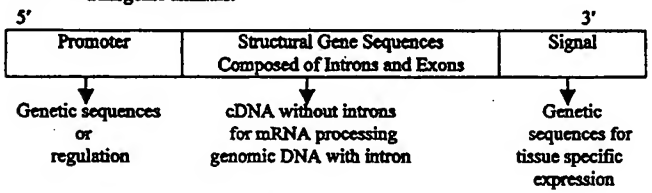
Transgenic technologies, however, are not without their own risks, particularly in the realm of the environment. For instance there is a concern that release of genetically modified organisms into an ecosystem may "contaminate" the wildtype members of the same or closely related species. This risk appears greatest in the case of plants, insects, and aquatic animals. In contrast, the environmental impact of transgenic livestock containing medically important genes is likely to be extremely low. Such valuable animals are produced and maintained in tightly regulated facilities and thus are unlikely to breed freely. For example, transgenic pigs that are designed to supply organs or tissues for xenotransplantation will not be accessible for purposes of meat or byproducts. This review describes recent revolutionary advances in the techniques needed to create genetically altered animals with medically and commercially valuable

characteristics. These exciting discoveries are described in the context of basic principles of cell and molecular biology.

II. INTRODUCTION

Every cell of every living animal contains tens of thousand of genes that are carefully packaged into the cell nucleus. All genes are built from double stranded DNA sequences and these sequences, in turn, are organized into functional cassettes which encode different types of information (Fig. 1).

Figure1. Schematic diagram of a fusion gene used for production of transgenic animals.



NOTE: The fusion gene can also contain protein binding elements, enhancers and repressor sequences (cis-Acting genetic elements)

For instance, the coding information within the genes is transcribed into single strand of RNA. Additional DNA sequences in the promotor element, usually located at the 5' end of a gene, are not transcribe into RNA but serve to bind specific proteins which, in turn, regulate the rate at which RNA is transcribed from the adjacent coding strand of the DNA double helix. Sequences at the 3' end of the gene act to limit the length of the transcribed RNA

Surprisingly, the information encoded within the central portion of the gene is actually organized in pieces, known as exons, interspersed with non-informational sequences, known as introns (Fig. 1). The primary RNA transcript synthesized from the DNA template undergoes a process known as splicing. Splicing stitches the informational pieces of the primary RNA transcript together into a secondary RNA molecule known as a messenger RNA (mRNA). This arrangement for archiving and decoding genetic information seems unnecessarily complex, but has greatly enhanced the frequency of new gene creation via exon shuffling over the course of evolution.

Once a mRNA molecule has been fully processed it leaves the cell nucleus and enters the surrounding cytoplasm where it is either stored for future use or is translated into a protein. Unlike DNA and RNA which are composed of nucleic acids, proteins are built by linking amino acids together into long chains (there

are twenty different amino acids) and then folding these chains into complex three dimensional structures. Proteins are functionally active molecules that provide the infrastructure that cells need to remain alive. Many proteins are considered "housekeeping" proteins because they are present in all living cells and are required to carry out the biochemical and structural process characteristic of life. In addition, each type of cell transcribes its own characteristic set of genes and the resulting messages are translated into cell specific sets of proteins. Thus cells from different organisms differ in terms of the genes that they possess, while cells within an organism often differ in terms of the genes that they express.

Humans have been deliberately altering the genetic composition of plants and animals ever since the agriculture revolution began and wild beasts were domesticated more than ten thousand years ago. Until recently however these genetic manipulations proceeded relatively slowly because they depended on breeding, inbreeding, and crossbreeding of genetic variants that arose as spontaneous mutations within a breeding population. The most famous of such breeding experiments were carried out by Gregor Mendel in 1867 (Mendel, 1966). In fact, Mendel was able to describe the basic laws of genetics by inbreeding and crossbreeding naturally occurring variants of peas. Similarly, animal breeding experiments and information about animal breeds were key parts of the evidence that Charles Darwin amassed to support his theory of The Origin of Species (1962). But despite their brilliant insights, neither Mendel nor Darwin knew anything about the biochemical basis or molecular basis of inheritance or mutagenesis.

Not only do we now know that DNA is the key informational molecule of life, but the ongoing revolution in molecular biology has made it possible to alter the sequence of DNA, create and select useful genetic alterations, and add genes from one species to those of another. The later technique is called transgenesis and traditionally involves insertion of genes into, or deletion of genes from a living embryo. Under optimal circumstances, the new genes are found in all cells of the embryo, including those that eventually become sperm and eggs, the gametes for the next generation. Transgenic animals are valuable either because they produce a protein of interest, or because their cells, organs, or whole bodies can be utilized for medical purposes or for food.

Until recently methods for creating transgenic animals were relatively inefficient and inaccurate. The investigator could not be sure that an added gene would insert and function correctly in the nucleus of the recipient cell. These limitations meant that in order to obtain new animals with the desired genetic traits a large number of transgenic animals had to be generated and tested, and most likely sacrificed. Such an uncontrolled approach is costly and not necessarily effective, particularly if applied to large farm animals.

Cloning, the latest breakthrough in animal breeding, offers an efficient approach to making transgenic animals. Cloning permits the nucleus from a body (somatic) cell, or a cell grown in a petri dish, to be reprogrammed and thereby to direct the development of an entire new offspring. If a gene is added

to the somatic cell before its nucleus is used for cloning, the cloned-transgenic animal that results is sure to carry the added gene in all of its cells, including every gamete. Moreover, because the cloning procedure can be repeated again and again (at least theoretically) an entire herd of genetically identical transgenic animals can be created in a relatively short period of time, without the need for inbreeding. The scientific advances leading up to this exciting new technology are described later in this article.

III. TRANSGENESIS

Via Microinjection of Message RNAs. Prior to isolating a gene and introducing the gene into the genome via microinjection, studies involving injection of mRNA into the cytoplasm of oocytes and fertilized eggs were carried out to determine the half-life (stability) of the mRNA, as well as the mechanism of protein translation and post-translational modification (Gurdon, 1973; Palmiter et al, 1978; Brinster et al., 1980). These investigations established that frog oocytes (Larkey et al, 1977) and fertilized mouse zygotes (one-cell embryos) (Brinster et al, 1981a; Ebert et al, 1983) could translate globin and ovalbumin mRNAs injected into the cytoplasm, even though these mRNAs did not originate from the nucleus of the recipient cell.

Subsequent experiments were performed to study the rate of protein production by labeling the protein product with a radioactive amino acid. The α-globin that was translated from the rabbit mRNA was measured by immunoprecipitation. These studies showed that there were significant differences in the ability to synthesize and secrete the protein from the Xenopus oocyte (Larkey et al, 1977) compared to the mouse oocyte (Brinster et al, 1980). Moreover, studies designed to compare the translation of microinjected foreign mRNA into oocytes versus fertilized egg showed significant differences between these two developmental stages, primarily in terms of the stability of the mRNA with similar translational capabilities (Paynton ,et al, 1983). These initial experiments were cornerstone to subsequent experiments designed to create genetically modified animals via direct injection of genes into the early developing embryo.

Via Microinjection of Mitochondria. As discussed in the introduction, most of the DNA of a cell is found within its nucleus where it is cleverly packaged in very long linear molecules known as chromosomes. Additional, much smaller circular DNA molecules are found in the numerous mitochondria of the cell. Mitochondria are subcellular organelles that play a critical role in energy metabolism and variations in mitochondrial DNA (mtDNA) are known to affect both the development and the phenotype of organisms. The mitochondria of a new embryo are derived from the population of dividing mitochondria of the egg, rather than the non-dividing mitochondria of the sperm. Thus, mitochondria are said to be maternally inherited (Dawid et al., 1972; Ebert et al, 1981). Studies were designed to further investigate this theory by microinjecting intact mitochondria from male germ cells and somatic cells into

the fertilized mouse zygotes. Analysis of the mitochondrial DNA indicated that it was maternal (Gyllensten et al, 1985). The data therefore support the conclusion that mtDNA is maternally inherited.

Via Microinjection of Genes into Fertilized Eggs. Efforts to create transgenic animals by direct injection of foreign DNA into the cell nucleus (Fig. 2) began in several laboratories using several organisms (see reviews Palmiter, 1986; Ebert et al., 1993) and eventually resulted in definitive evidence that specific genes can integrate into the host cell genome in a predictable fashion (Brinster et al., 1981b; Gordon et al, 1985). The variables that most affect the efficiency of integration have been documented (Brinster et al., 1985). While microinjection experiments clearly demonstrate that exogenous DNA can integrate into the host genome, the actual process of integration remains mysterious. Neither the site, the timing, nor the number of genes integrated can be accurately predicted using current microinjection approaches. These uncertainties limit the utility and substantially increase the costs involved in making transgenic animals.

IV. TRANSGENIC TECHNOLOGIES THAT GENETICALLY MODIFY ANIMALS

The initial experiments to incorporate foreign genetic information was pioneered by Brinster et al. (1981) and Gordon et al. (1981). Although microinjected DNA was not shown to be expressed, the genes were integrated in the genome and remained stable throughout development. Modification of fusion genes ultimately corrected this problem when the appropriate promoter, structural and signal sequences were added to effectively allow for the expression of the foreign gene.

Transgenic Animal Models. The ability to phenotypically change animals was demonstrated by the production of transgenic mice expressing the rat and human growth hormone (Palmiter et al., 1983; Hammer et al., 1985; Ebert et al, 1988). This led to a proliferation of the technology to many areas of research, both in laboratory animal models, agricultural alternatives, and production of pharmaceutical products for a more efficient supply of therapeutical proteins compared to the in vitro cellular expression process. A few examples of this successful research are presented.

The transgenic technology has identified the elements of a given gene that are required to allow for appropriate expression of the gene and its specific expression in particular organs or tissues. One of the initial experiments to show that foreign genes can be expressed in selective tissue was reported by Palmiter et al (1983). These experiments showed that an artificially modified gene containing the metallothionein promoter and a structural gene (GH) produced a foreign gene product in specific tissues such as liver. The concept that one could generate a fusion gene to specifically be expressed in a selective tissue or organ generated a series of reports which showed specific expression of modified fusion genes (Bedell et al., 1997). Examples of the use of transgenic

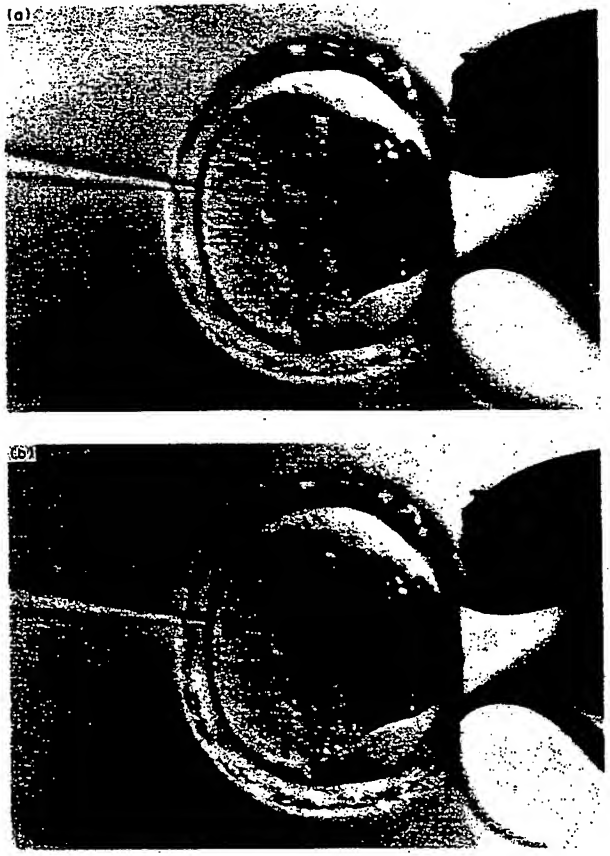


Figure 2. Top panel is a pig zygote that was centrifuged in an Eppendorf centrifuge for 4 min at 13 000 x g, at room temperature. The male pronucleus is being injected. Note the lipid inclusions are cleared from the central portion of the cytoplasm during centrifugation. The bottom panel is the same embryo after injecting approximately 2 pl of solution. The embryo is approximately 70 μm in diameter

animals to identify particular gene sequences within a gene that are required for tissue specific expression are presented.

The Use of Transgenic Animals for Understanding Genetic Control Mechanisms. Experiments have been specifically designed to study the required genetic elements responsible for the accurate expression of a gene. For instance, there are two distinct promoter elements responsible for the expression of proenkephalin in mice and rats. One is responsible for expression in spermatogenic cells (Zinn et al., 1991) and the other is responsible for expression primarily in the brain. Experiments were performed to differentiate the promoter regions and to identify the elements that are required for specific targeted expression of the gene product; genomic DNA was used to accomplish the molecular dissection of the gene sequences required for complete and appropriate expression of the gene. Using transgenic technology, the testes specific region within the gene was identified and a fusion gene introduced into mouse zygotes which ultimately produced offspring that showed only the testes expression and not in other tissues (Zoya, et al., 1993). These experiments revealed that the minimal rat proenkyphalin germ line promoter was localized to a 116-bp region encompassing the transcriptional start site region. These experiments also identified a proximal 51-bp sequence located in the 5'-flanking region that is also required for germ line promoter activity during spermatogenesis and production of early haploid spermatids (Liu et al., 1997). This example, one of many reports, illustrates the power of transgenic technology to identify specific gene sequences that are required for tissue specific gene expression in a whole organism. Knowledge at this level of detail is needed for construction of transgenic animals that express valuable proteins in specific tissues, such as the mammary gland, at levels that the animal can physiologically tolerate.

V. TRANSGENESIS VIA ADDITION OF GENETICALLY ALTERED EMBRYONIC STEM CELLS TO EARLY EMBRYOS

Direct injection of genes into the nucleus of a fertilized egg, as discussed above, is not the only strategy for creating a transgenic animal. An alternative approach involves isolating cells from very early embryos, genetically modifying them, and then returning them to another embryo. Genetic modification is accomplished by growing the cells in cell culture under conditions that prevent them from differentiating. Under these conditions new genes can be added (knocked-in) or endogenous genes can be removed (knocked-out). When the modified cells are returned to the embryo they join those of the intact embryo, which now develops into a chimeric animal composed of cells from two entirely separate sets of parents. This technique works because the cells of very early embryos are stem cells that can develop into virtually any type of cell in the body, even germ cells. However, only a small fraction of chimeric animals actually possess genetically modified sperm or eggs. This uncertainty means that many chimeric animals have to be created,

tested, and bred to identify those which contain genetically modified sperm or eggs along with normal sperm or eggs. Thus, a fraction of the second generation animals created by embryonic stem cell transfers are genetically modified in every cell and breed true.

Embryonic stem cell transfer has been/is being extensively exploited to create both knock-in mice (having one or more added genes) and knock-out mice (missing one or more genes). Homozygous knock-out animals (inbred strains missing both copies of a gene) are particularly intriguing experimental animals because they provide new insights into gene function, particularly during early development, and can be utilized as models for genetically inherited diseases. Despite all of this knowledge, construction of a transgenic mouse via embryonic stem cell transfer remains an expensive and time consuming project due to the inherent uncertainties at each step in the process. Embryonic stem cell transfer has thus far proven useless for construction of transgenic animals of any other species. The primary reason for this is that embryonic stem cells capable of developing into germ cells have never been isolated in any species other than the mouse. Cloning offers a new technology for overcoming this problem.

VI. CLONING

Multicellular organisms are highly coordinated aggregates of organs, each of which has its own function and specialized types of cells. At least some of the genes transcribed in these different cell types are cell-type specific. Differentiated cells therefore differ in terms of the proteins they synthesize, the shapes they acquire, and functions they carry out. At least two molecular mechanisms could account for how different cell types arise as the organism develops from a fertilized egg: A) The process of cell differentiation could involve irreversible developmentally programmed changes in DNA sequence or modification. According to this scenario, only the primordial germ cells of an organism (those that give rise to sperm and eggs) would retain a full complement of genes, while somatic cells of the body would discard, rearrange, or otherwise alter portions of their DNA as they develop and differentiate. Genetic changes like this are known to take place in some single cell organisms and insects, as well as antibody producing cells of higher organisms; B) Alternatively, germ cells and somatic cells could have virtually the same DNA composition, but could differ in terms of how their genes are packaged, as wells as how they are epigenetically modified. According to this scenario the processes of cellular development and differentiation would largely depend on DNA-protein interactions. Under appropriate circumstances interactions can be reversed or reprogrammed. A very large body of molecular information amassed over the last four decades supports the view that DNA-protein interactions are the key to gene packaging and gene transcription. Additional evidence shows that epigenetic changes in DNA methylation and telomere length take place during cellular differentiation and aging. However, molecular

and biochemical experiments alone cannot directly demonstrate whether or not such changes are reversible or irreversible since these are properties of genome function in living cells undergoing development and differentiation.

Embryologists have long attempted to definitively establish whether or not development is an irreversible process by transplanting the nuclei of somatic cells into enucleated eggs. This experimental approach, known as nuclear transplantation is the basis for cloning. The transplanted nucleus is taken from the somatic cell, known as the nuclear donor and is injected or otherwise transferred into the egg, known as the nuclear recipient. The eggs own nucleus is either destroyed or removed prior to fusion with the donor nucleus, thereby guaranteeing that the resulting embryo is a clone of the somatic cell donor. The nuclear transplantation approach predicts that the recipient egg will develop into a completely normal embryo if the donor nucleus has not undergone irreversible changes during its own differentiation. Conversely, if somatic cell differentiation does involve irreversible changes, the recipient egg is expected to achieve only incomplete or limited development in response to the transplanted nucleus. While the logic of the nuclear transplantation technique is clear, its execution is technically demanding and is complicated by the fact that it is often difficult to determine whether a particular embryo fails to develop normally because the donor nucleus was damaged or inadequately reprogrammed for development before or after it was placed into the recipient egg, or because the donor nucleus was , indeed, irreversibly altered during its own development and differentiation process.

Attempts to create cloned animals by transplantation of embryonic and somatic cell nuclei date back to the beginning of the twentieth century (Spemann, 1938). The usual procedure involves transplantation of a nucleus into an unfertilized egg that has been activated and thus is already started on its path toward new DNA synthesis and cell division. A great many experiments based on this approach demonstrated that the best results were obtained by transfer nuclei recovered from the undifferentiated cells of early embryos, or from rapidly dividing differentiated cells. These experiments proved again and again that the nuclei of very early embryonic cells are, indeed, capable of directing complete normal development and are therefore totipotent in their genetic capacities. In contrast, the nuclei of more differentiated cells typically exhibited significantly more limited capacity to direct normal development and are therefore said to be pluripotent, i.e. capable of generating many, but not all types of cells (DiBerardino, 1997). Despite these limitations, two sets of experiments suggested that the hurdles to somatic cell cloning were not insurmountable. The first experiments were carried out by John Gurdon in 1960s. He succeeded in creating two frogs from the nuclei of two tadpole intestinal cells (Gurdon, 1962; Gurdon et al., 1966). Although the experiment was never repeated it clearly suggested that somatic cell nuclei could be fully reprogrammed. The second set of experiments were carried out by Marie Di Berardino and her colleagues (1986) and point the way toward a method for reprogramming nuclei. These investigators demonstrated that the nuclei of

triploid erythrocytes, which are fully differentiated quiescent cells, could be decondensed and reactivated if they were injected into oocytes on there way to maturing into eggs. Red cell nuclei treated in this way were able to support advanced tadpole development. These seminal experiments clearly suggested that cellular differentiation is difficult but not impossible to reverse. Together they held out the promise that complete genomic reprogramming and reproducible creation of cloned offspring would one day be achieved, if the optimal conditions for choosing, preparing, and manipulating somatic cell nuclei could be found.

As all the world now knows, Ian Wilmut and his team of scientists in Scotland succeeded in creating the first cloned mammals (1997). Since that time, cloned mice (Wakayama et al., 1998), cows (Cibelli et al., 1998), and goats (Baguisi et al., 1999) have been created by other teams of scientists working in Hawaii, Massachusetts, and Japan. While the nuclei used to create all of these animals have been recovered from several types of somatic cells, the method for preparing and handling them has been very similar in all cases.

The key steps in the method for cloning from somatic cell nuclei were actually invented and patented in 1993 by one of us, Lawrence Wangh and his team of scientists at Brandeis University in Waltham Massachusetts. Rather than use the nuclei of dividing cells, we chose to use nuclei of quiescent, or non-dividing cells because quiescent cells, like sperm cells, are very homogeneous and are poised to rapidly reenter the cell cycle in response to appropriate stimuli. The critical steps for genetic reprogramming consist of freeing the nucleus from the structural constraints imposed by the cytoskeleton of the cell and then activating the nucleus for efficient replication and renewed development. As we first described (Wangh et al., 1995), the actual procedure is relatively simple and extremely flexible, once the logic of nuclear reactivation is understood. In fact, the steps involved in nuclear reprogramming can be observed and optimized in a test tube, prior to actually placing the nucleus into an unfertilized egg.

As the preceding discussion indicates the repeated creation of cloned animals from the nuclei of several types of somatic cells is of basic scientific interest because it settles one of the central questions of developmental biology. There is no longer any doubt that cellular differentiation fundamentally depends on protein-DNA interactions and epigenetic changes in DNA structure that can be reversed under specific circumstances. However, the importance of cloning does not stop there. Because cloning is a technique that can be applied to many species, including humans, cloning also raises a host of ethical, philosophical, legal, and religious questions and paradoxes concerning the meaning of self identity and individuality (Nussbaum and Sunstein, 1997). Finally, unlike many other scientific discoveries that have taken a relatively long time to move from the basic research laboratory into the arena of biotechnology, cloning has immediately found uses in the construction of transgenic animals.

Transgenesis Via Cloning. The logic for creating transgenic animals via cloning is outlined in Figure 3.

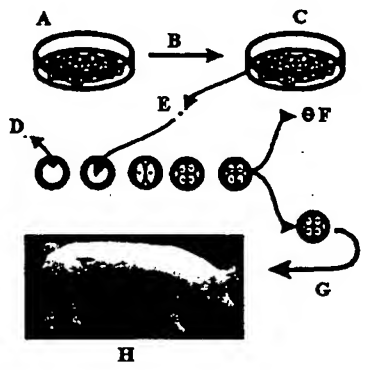


Figure 3. Production of transgenic animals through cloning via nuclear transplantation.
A. Pig Cells in Culture. Add Human Gene
B. Gene Insertion/Gene Expression
C. Transgenic Pig Cells with Human Gene
D. Remove Nucleus from Egg
E. Transplant Nucleus of Transgenic Cell
F. One Cell Removed for Genetic Analysis (Optional)
G. Transfer Embryo to Surrogate Female
H. Transgenic Animal

Cells recovered from an embryonic, fetal, or adult donor are placed in culture and encouraged to grow. Genes that encode the information need to synthesize commercially valuable proteins are added to the growing cells. Cells that have taken up and integrated the added genes are identified and selectively separated from those that have not. Once a small group of cells has been selected because they have the desired gene, the cells are starved for growth factors in order to drive them into quiescence. As explained above, the nuclei of quiescent cells are well suited for nuclear transfer because they are uniform and poised to reenter the cell cycle if they contact the cytoplasm of an unactivated egg. In a functional sense, the quiescent state of the donor nucleus is matched to the quiescent state of the recipient cytoplasm of the egg. Because the recipient egg is arrested in metaphase II of the cell cycle, the nuclear envelope and matrix of the donor nucleus break down in the cytoplasm of the unactivated egg. This alteration in nuclear structure serves to reorganize and genetically reprogram the

donor nucleus. Thus when the recipient egg is subsequently activated to enter the first cell cycle, the donor nucleus is ready to decondense, reactivate and form a new fully functional nucleus capable of complete DNA replication and normal development. The recipient eggs' own nucleus is removed or destroyed, either before or after the donor nucleus is added to the recipient egg, thereby guaranteeing that the egg's genes are not present in the cloned embryo. If the donor nucleus comes from a transgenic cell, as outlined in Fig. 3, the new embryo is a transgenic clone. This means that every cell of the animal arising from this embryo is sure to carry the added transgene.

A commercially important variation on this scheme involves removal or replacement of one gene with that of another prior to creation of a cloned animal. Such animals are known as "knock-out" transgenics. The logic for a gene "knock-out" is very similar to that of adding a gene through "knock-in" technology. The undesired gene is eliminated from the donor cells while they are in cell culture and the nucleus of the modified cell is then used to create the cloned animal.

Construction of "knock-out" animals via cloning has not yet been described, but "knock-out" mice are now routinely constructed via addition of modified embryonic stem cells to the inner cell mass of early mouse embryos (see above). The resulting chimeric embryos are then grown up into first generation animals that may pass on the hemizygous "knock-out" condition to some of their offspring. If two such second generation animals are mated, one quarter of their offspring in the third generation are expected to be missing both copies of the missing gene. Depending on what role the eliminated gene plays in normal development and adult life, these homozygous "knock-out" animals may or may not grow and behave normally. Thus, homozygous "knock-out" mice are proving very useful for learning about functions of many genes, particularly those that mimic human diseases.

As outlined above, construction of homozygous "knock-out" animals requires the availability of embryonic stem cells, as well as three generations of breeding. Embryonic stem cells are not currently known for valuable domestic animals like the pig and the cow and raising and breeding of these animals if very expensive. Construction of "knock-out" animals via cloning does not use an embryonic stem cell and also guarantees that all of the first generation cloned animals are heterozygous for the eliminated genes. These animals can then be bred to each other to generate a homozygous "knock-out" animal in the second generation. Alternatively, fetal cells from first generation "knock-out" animals can be placed in culture and selected again and then can be used to generate a homozygous "knock-out" cloned embryo, even before the first generation "knock-out" animals reaches adulthood.

The above strategies for efficient construction of "knock-in" and "knock-out" commercially valuable cloned animals are still in their experimental infancy. But, in contrast to the pre-cloning era, they are no longer in the realm of science fiction. The most difficult and intellectually interesting step, cloning itself, is now a demonstrated reality. What lies ahead is a period of intensive

and secretive competition among biotechnology companies trying to exploit and expand the new possibilities inherent in this breakthrough. Some of the commercially and socially valuable products on the drawing boards are discussed below. Before describing these, however, there is one additional technology, transgenesis via gene-sperm-transplantation, that warrants consideration because it may soon render transgenesis via cloning obsolete, at least for the generation of "knock-in" animals.

Transgenesis Via Gene-Sperm-Transplantation. The sperm is the natural vector for delivering genes into an egg. Addition of genes to sperm or sperm nuclei prior to fertilization is an obvious alternative approach to construction of "knock-in" animals, and should be far more efficient than somatic cell cloning or direct injection of genes into a zygotic pronucleus. The sperm nucleus does not have to be reprogrammed, nor does the oocyte's nuclear material have to be removed. Naturally, if sperm are used to deliver new genes into an egg, the resulting offspring are not clones of either parent, but this is usually of little consequence for most cases of transgenesis.

Direct addition of genes to intact sperm, followed by in vitro fertilization has been attempted over the years in a variety of animals, including mice, fish, and chickens. Evidence demonstrating the presence of the added DNA in first generation offspring has been reported (Lavitrano, et al., 1989) but the reliability of this technique is far more controversial. Apparently much of the DNA added directly to sperm, like DNA injected directly into the cytoplasm of an egg is either degraded or discarded during the course of early embryonic development (Perry, et al., 1999).

An alternative approach to gene-sperm-transplantation borrows from the *in vitro/in vivo* cloning strategy first described by Wangh et al. (1995) for cloning from somatic cell nuclei. In this case sperm nuclei, like other quiescent cell nuclei, are stripped of their surrounding membranes and cytoskeletal constraints and are decondensed in vitro egg cytoplasm that does not support new nuclear envelope formation and DNA synthesis. The exogenous DNA is added directly to the decondensed nucleus and is even encouraged to integrate into the sperm genome in vitro by introducing frequent breaks and nicks that are later repaired in the egg. Because these steps are carried out in the test tube, each can be manipulated and optimized before the sperm nucleus is injected into the egg. Sperm-gene-transplanation using decondensed sperm nuclei has become the method of choice for construction of transgenic frogs over the last few years (Kroll and Amaya, 1996).

Gene-sperm-transplantation may be slightly more difficult for production of transgenic mammals because the sperm of mammals contain their own unique nuclear proteins, call protamines, that stabilize the sperm genome with extra crosslinking, known as thiol bridges. Nevertheless, we have established conditions for dissolving these bridges and decondensing mammalian sperm nuclei and are well on our way to construction of transgenic pigs, rats, goats, and other valuable animals using this approach (Neuber al., 1999). Very recently Perry et al. (1999) have demonstrated permeabilization of the sperm

plasma membrane is the only in vitro treatment required for efficient production of transgenic mice. It remains to be seen whether this very simple treatment will be sufficient to achieve nuclear reactivation of sperm in cows, pigs, sheep and other domestic species which are known to be more resistant to nuclear reactivation.

Permabilized and partially decondensed sperm nuclei with added genes are no longer viable in the sense that they do not swim and cannot penetrate an egg on their own. Injection of their nuclei directly into the unfertilized egg circumvents this difficulty (Kimura and Yanagimachi, 1995). At first view it may seem remarkable that a sperm, which normally only enters an egg after undergoing an elaborate set of preparatory steps known as capacitation and acrosome activation, can initiate development after being injected directly into an egg. Even more remarkably, this phenomenon was first described in humans without prior experimentation in any mammalian experimental system. The technique is called intracytoplasmic sperm injection, ICSI, and was inadvertently invented in 1992 by Belgian physicians who were treating infertile couples by injecting sperm adjacent to the plasma membrane surrounding the egg (Van Steirteghem et al., 1993). In one case the needle slipped and the entire sperm cell was injected directly into the egg cytoplasm. To the surprise of the physicians and embryologists alike, ICSI results in a very high frequency of normal fertilizations and has now become the method of choice for giving many infertile couples the healthy baby they so ardently desire.

VII. APPLICATIONS OF THE TRANSGENIC TECHNOLOGY

Production of Pharmaceuticals in Milk. The ability to combine major gene segments for directed expression of a foreign gene in targeted tissues, offers the possibility to commercialize the technology in transgenic livestock (Fig. 4). Initially it was targeted toward growth enhancement and leaner meat products (Hammer et al., 1985). However, due to unanticipated problems with the transgenic animals, that expressed the growth hormone gene in a non-regulated vector system, the growth and leanness objectives have not produced a commercially viable product (Ebert et al., 1988). The technology has been successful in production of pharmaceutical proteins that could be expressed specifically in the mammary gland (milk) in order to efficiently produce large quantities of specific proteins and decrease the cost of producing these therapeutic proteins using *in vitro* cell culture technology. The transgenic technology rapidly moved toward the design of vectors that would direct the expression specifically to the mammary epithelium and ultimately produce a continual and *in vivo* production of selective proteins.

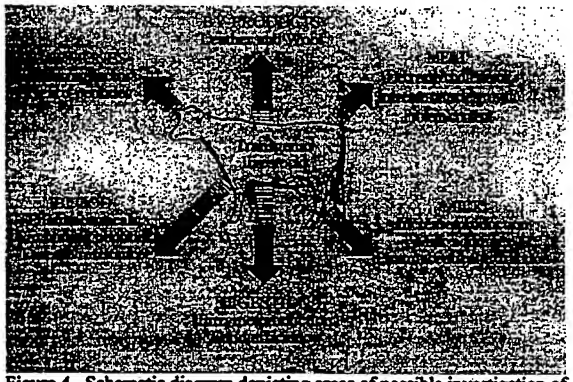


Figure 4. Schematic diagram depicting areas of possible investigation of domestic livestock using transgenic methodology.

The production of transgenic livestock (cattle, pigs, sheep, goats) was directed towards the production of transgene proteins specifically in the mammary gland for its secretion into milk. Mouse models were initially produced in order to reduce this idea to practice. Over the years it became obvious that hybrid genes were effectively expressed in the mammary gland with various milk specific promoters that regulate mammary specific proteins, such as: murine whey acid protein (Gordon et al., 1987), rat β-casein (Lee et al., 1989), bovine alphaS1 (Meade et al., 1990). In addition, using these promoters allowed for significant quantities of heterologous protein to be produced in mouse milk (see Review, Maga et al., 1995).

The success with transgenic mice quickly moved toward the production of transgenic livestock that could produce large volumes of milk for possible commercial value. This renewable source of milk could supply pharmaceutical peptides used in human therapy such as human tissue-type plasminogen activator (Ebert et al., 1991), and alpha-1-antitrypsin (Wright et al., 1991). The transgene is specifically expressed and the concentration of the heterologous protein can range from undetectable levels to grams per liter depending on where the fusion gene was integrated in the genome. The upper limit of production of a pharmaceutical protein is thought to be a more economical source of valuable therapeutic proteins when compared to in vitro cell cultures. The purification and characterization of the heterologous protein shows that the protein can be harvested in large quantities and enzymatically active (Denman et al., 1991). It is yet to be proven whether a heterologous protein from transgenic animals using mammary production is biologically active in living animals or humans.

Transgenic Livestock With Increased By-product Value. As shown in Fig. 4,

experiments are also being conducted to utilize the transgenic technology to add a value added component to industrial by-products of economically important species. Some of the recent experiments show promise toward this goal. Wool and leather are by-products that may be affected by genetic engineering. The basics in the physiology of its production would lead to the discovery of selected genes that may improve the growth and quality of these commercially important products.

Several approaches to alter structural proteins which are important to the quality of the by-product have been proposed (Ward, 1982). For example, the limiting elements in the production of these products may be altered by introducing genetic information to enhance growth and quality. Early studies have shown that an increase in cysteine in sheep resulted in an increase in wool growth (Reis, 1979). Using transgenic technology sheep were generated to increase expression of insulin-like growth factor that was driven by a mouse ultra-high-sulfur keratin promoter (Damak, et al., 1996). Although a modest improvement in wool characteristics were evident, the technology shows that fiber qualities can be altered with genetic manipulation through transgenesis.

Transgenic Livestock for Xenotransplantation Organs. The ability to direct selective heterologous gene products to transgenic animals offers the possibility to produce large domestic species that express proteins that when appropriately expressed and functional would potentially eliminate some of the hurdles involved in organ rejection from donor to recipient. It is well known that the hyperacute rejection is the major reason why organs from a non-human species are rapidly rejected by organ transplant recipients (Dalmasso, et al., 1992) . The transgenic technology offers a unique opportunity to genetically alter the surface antigens on organs in order to minimize immunological reactions that are responsible for the xenograft rejection. For instance, modification of the surface antigens via the expression of proteins that may inhibit complement activation has been successful in inhibiting xenograft rejection in the pig to primate (Laambrigts, et al., 1998).

Due to their anatomical and physiological similarities, pig organs are the tissues of choice for transplantation into humans. Transgenic experiments presently ongoing have successfully introduced the human α1,2-Fucosyltransferase into pigs, thus modifying the cell surface carbohydrate phenotype that resist the human serum-mediated cytolysis (Costa, unpublished). These first generation experiments hold out the promise of very exciting medical advances in the future. Currently tens of thousands of desperately ill patients in the United States go unserved because the organ they need cannot be located. It seems likely that within the next decade advances in pig transgenesis will overcome this problem.

While the above scheme may appear fantastical, cloning offers even more remarkable possibilities. For instance, scientists as the Geron Corporation are actively pursuing the possibility of partial self-cloning for therapeutic purposes (Solter and Gearhart, 1999). Under this scheme the nucleus of a cell taken from a patient would be transplanted into an enucleated human egg for the purpose of growing a human embryo. The embryo, in turn, would be used as a source of embryonic stem cells. These pluripotent cells, in turn, would be used to create new tissues or a new organ, which would then be transplanted back into the patient. The experimental details required to convert this scheme into a reality remain to be worked out, but the key steps are clearly feasible. The question remains however, is this form of medicine desirable? Now is the appropriate time for thorough ethical debate about this impending technology.

VIII. CONCLUSIONS

It is now clear that the first decade of the 21st Century will see the rapid mastery and refinement of transgenic and cloning techniques in many commercially valuable domestic species, as well as increasing exploitation of these procedures to alleviate human medical problems. Without question the overriding problems of the 21st Century will be feeding the burgeoning human population, while decreasing environmental resources as little as possible. We predict that transgenic technologies will be critical to rapid production of food-efficient and disease-resistant breeds of animals that contain genes normally found in non-domesticated species. Transfer of these genes into domesticated species would be impossible by conventional breeding. The emerging revolution in animal transgenesis may prove similar to the "green revolution" that staved off food shortages predicted in the 1960's. Public debate over the uses of these technologies is appropriate and will be beneficial, provided it is based on accurate information and a clear understanding of the underlying biology. We hope that this review serves to stimulate thoughtful and productive dialogue between reproductive biologists, environmental scientists and people who are interested in advancing technology for the benefit of humankind.

FIGURES

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Cloned Calves from Chromatin Remodeled In Vitro[1]

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ABSTRACT

We have developed a novel system for remodeling mammalian somatic nuclei in vitro prior to cloning by nuclear transplantation. The system involves permeabilization of the donor cell and chromatin condensation in a mitotic cell extract to promote removal of nuclear factors solubilized during chromosome condensation. The condensed chromosomes are transferred into enucleated oocytes prior to activation. Unlike nuclei of nuclear transplant embryos, nuclei of chromatin transplant embryos exhibit a pattern of markers closely resembling that of normal embryos. Healthy calves were produced by chromatin transfer. Compared with nuclear transfer, chromatin transfer shows a trend toward greater survival of cloned calves up to at least 1 mo after birth. This is the first successful demonstration of a method for directly manipulating the somatic donor chromatin prior to transplantation. This procedure should be useful for investigating mechanisms of nuclear reprogramming and for making improvements in the efficiency of mammalian cloning.

early development, embryo

INTRODUCTION

Limitations to the application of mammalian cloning technology by nuclear transplantation (NT) are low rates of embryonic development, high rates of pregnancy loss, and low survival of cloned offspring [1–3]. Substantial effort has been put toward characterizing defects in cloned embryos and offspring; however, information gathered from these studies [2–4] has not yet yielded any novel approaches for improving survival of clones.

Extracts from gametes or somatic cells have been used to investigate the dynamics of the cell nucleus at fertilization or during the cell cycle [5, 6]. Notably, extracts from mitotic cells have been shown to support disassembly of exogenously added nuclei, including nuclear envelope breakdown and chromosome condensation [7–9]. In vitro nuclear disassembly is compatible with subsequent reconstitution of functional nuclei [9]. Moreover, somatic cell extracts containing nuclear and cytoplasmic components have been developed to alter chromatin organization and gene expression in exogenous nuclei [10] or in permeabilized somatic cells [11].

The objective of this study was to determine whether cloned animals could be produced after manipulating the somatic donor nucleus in vitro prior to transfer into a recipient oocyte. We report that disassembly of somatic nuclei in a mitotic extract followed by transfer of the resulting condensed chromatin into oocytes enhances nuclear remodeling. Chromatin transfer (CT) is compatible with development to term and shows trends of improved viability of cloned animals.

MATERIALS AND METHODS

In Vitro-Produced Embryos

In vitro fertilization was performed, and embryos were cultured as described [12, 13].

Nuclear Transplantation and Oocyte Activation

NT was carried out by fusing donor bovine fetal fibroblasts to enucleated oocytes [1, 13]. Metaphase stage NTs (M-NTs) were performed in nocodazole-containing media (1 μg/ml) using donor fibroblasts synchronized in M phase with 1 μg/ml nocodazole for 18 h. Recipient oocytes were activated at 28–30 h postmaturation (hpm) with 5 μM calcium ionophore for 4 min followed by 10 μg/ml cycloheximide (CHX) and 2.5 μg/ml cytochalasin D for 5 h and washed, and embryos were cocultured with mouse fetal fibroblasts [13]. For CHX treatment, oocytes were activated as above and embryos were cultured with 2.5 μg/ml CHX for another 9 h before culture. For actinomycin D (ActD) treatment, oocytes were activated as above except that 5 μg/ml ActD was added to the 5-h CHX incubation step and embryos were maintained in 5 μg/ml ActD for another 9 h prior to culture.

Mitotic Extract

Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection, Bethesda, MD) were synchronized in mitosis with 0.75–1 μg/ml nocodazole for 18 h, harvested by mitotic shake off, and washed twice in phosphate buffered saline and once in cell lysis buffer (20 mM Hepes, pH 8.2, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM dithiothreitol, and a cocktail of protease inhibitors) [7]. Sedimented cells were resuspended in 1 volume of ice-cold cell lysis buffer, swollen on ice for 1 h, and Dounce-homogenized using a tight-fitting glass pestle. The lysate was centrifuged at 15 000 × *g* for 15 min at 4°C, and the supernatant (mitotic extract) was aliquoted, frozen in liquid nitrogen, and stored at −80°C. Fresh or frozen extracts were used without noticeable differences on the efficiency of nuclear breakdown.

Chromatin Transfer

In vitro-matured oocytes were enucleated at 20 hpm. Bovine fetal fibroblasts from confluent cultures were washed in Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS; Gibco-BRL; Invitrogen, Carlsbad, CA) and permeabilized by incubation of 100 000 cells in suspension with 31.2 U Streptolysin O (SLO; Sigma, St. Louis, MO) in 100 μl HBSS for 30 min in a 37°C H_2O bath. Permeabilization was assessed by uptake of the membrane impermeant DNA stain, propidium iodide (0.1 μg/ml). Permeabilized fibroblasts were sedimented, washed, and incubated in 40 μl mitotic extract containing an ATP-generating system (1 mM ATP, 10 mM creatine phosphate, and 25 μg/ml creatine kinase) for 45 min at 38°C. Aliquots were labeled with 0.1 μg/ml Hoechst 33342 to monitor chromatin condensation. At the end of incubation, the reaction mix was diluted with

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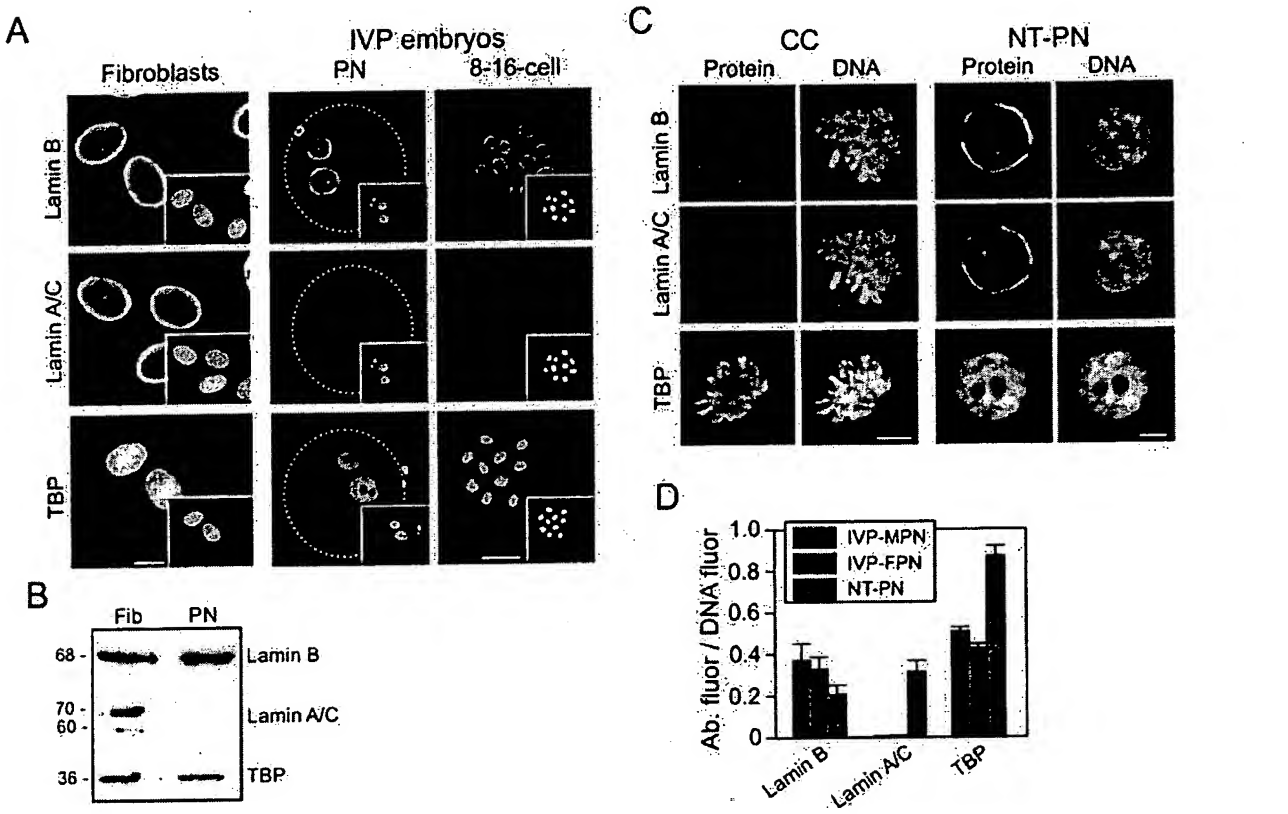

FIG. 1. Dynamics of fibroblast nuclei after NT. A) Immunofluorescence distribution of lamin B, lamin A/C, and TBP in bovine fibroblasts and IVP pronuclear (PN), and 8–16-cell stage bovine embryos. Insets = DNA labeled with Hoechst 33342; bars = (left) 10 μm, (right) 50 μm. B) Immunoblot of lamin B, lamin A/C, and TBP in fetal fibroblasts and pronuclear embryos. C) Immunofluorescence analysis of lamin B, lamin A/C, and TBP at condensed chromosome (CC) and pronuclear (NT-PN) stages in NT embryos. Bars = 10 μm. D) Immunolabeling intensity of indicated proteins in MPN and FPN of IVP embryos and in pronuclei of NT embryos (NT-PN). Data are expressed as mean ± SD ratio of antibody fluorescence over Hoechst 33342 (DNA) fluorescence intensity. More than 20 embryos/markers were analyzed in 3–5 replicates in (A, C, D).

500 μl Alpha MEM/10% fetal bovine serum (Gibco-BRL) containing 2 mM $CaCl_2$ for membrane resealing, and cells were cultured for 2 h at 38.5°C. Resealing was monitored by propidium iodide (0.1 μg/ml) uptake. Resealed cells were fused to enucleated oocytes, oocytes were activated at 28 hpm, and embryos were cultured as described for NT.

Embryo Transfer

NT and CT embryos were cultured to the blastocyst stage in vitro, and two embryos were transferred per synchronized recipient female. Pregnancies were monitored by ultrasonography, and C-sections were performed by independent veterinarians. All animal work was performed following a protocol approved by the Trans Ova Genetics (Sioux Center, IA) institutional animal care and use committee.

Immunological Procedures

Goat polyclonal antibodies against B-type lamins (referred to as "lamin B" in this paper), anti-lamin A/C monoclonal antibodies, and anti-TBP polyclonal or monoclonal antibodies were from Santa-Cruz Biotechnology (Santa Cruz, CA). Anti-AKAP95 antibodies were from Upstate Biotechnologies (Lake Placid, NY). Immunofluorescence analysis was performed as described [14]. Briefly, cells and embryos were settled onto poly-L-lysine-coated coverslips, fixed with 3% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and proteins were blocked in PBS/2% BSA/0.01% Tween 20. Samples each were incubated with primary and secondary antibodies (1:100 dilutions) for 30 min. DNA was counterstained with 0.1 μg/ml Hoechst 33342. Photographs were taken with a JVC CCD camera, and quantification of immunofluorescence intensity was performed using the AnalySIS software. When indicated, samples were extracted on coverslips with a cocktail of 1% Triton X-100, 1 mg/ml DNAse I, and 300 mM NaCl in Tris-HCl (pH 7.2) for 15 min prior to fixation and immunofluorescence analysis. For immunoblotting, protein samples (30 μg) were resolved by 10% SDS-PAGE, blotted onto nitrocellulose, and probed with indicated antibodies [14].

RESULTS

Behavior of the Donor Nucleus in Nuclear Transplant Embryos

We first investigated the dynamics of components of somatic nuclei during bovine NT. We examined the distribution of nuclear lamins, intermediate filament proteins of the nuclear envelope, and intranuclear skeleton. B-type lamins are ubiquitously expressed [15]. A-type lamins (which include the splice variants lamins A and C) are restricted to differentiated cells [15] and would not be expected to be expressed in preimplantation NT embryos. Lamins anchor nuclear membranes to chromatin and may promote nuclear expansion after nuclear reconstitution in vitro [16, 17]. Furthermore, mutations in the lamin A (*LMNA*) gene cause life-threatening diseases in humans [15], suggesting that lamins may be involved in the regulation of gene expression. Nuclear lamins are also essential for cell survival, as failure to assemble B-type lamins leads to cell death [18]. As a nonhistone component of chromatin and as a marker of the transcription machinery, the dynamics of the TATA-binding protein, TBP, a transcription factor for virtually all genes [19], was also analyzed. Perinuclear distribution of lamin B and colocalization of TBP with DNA in bovine fetal fibroblasts and in in vitro-produced (IVP) preimplantation embryos were consistent with observations in other species [20–22] (Fig. 1A). Lamin A/C was not detected during preimplantation development (Fig. 1A) as expected from a marker of differentiated cells. Immunofluorescence data were corroborated on Western blots (Fig. 1B).

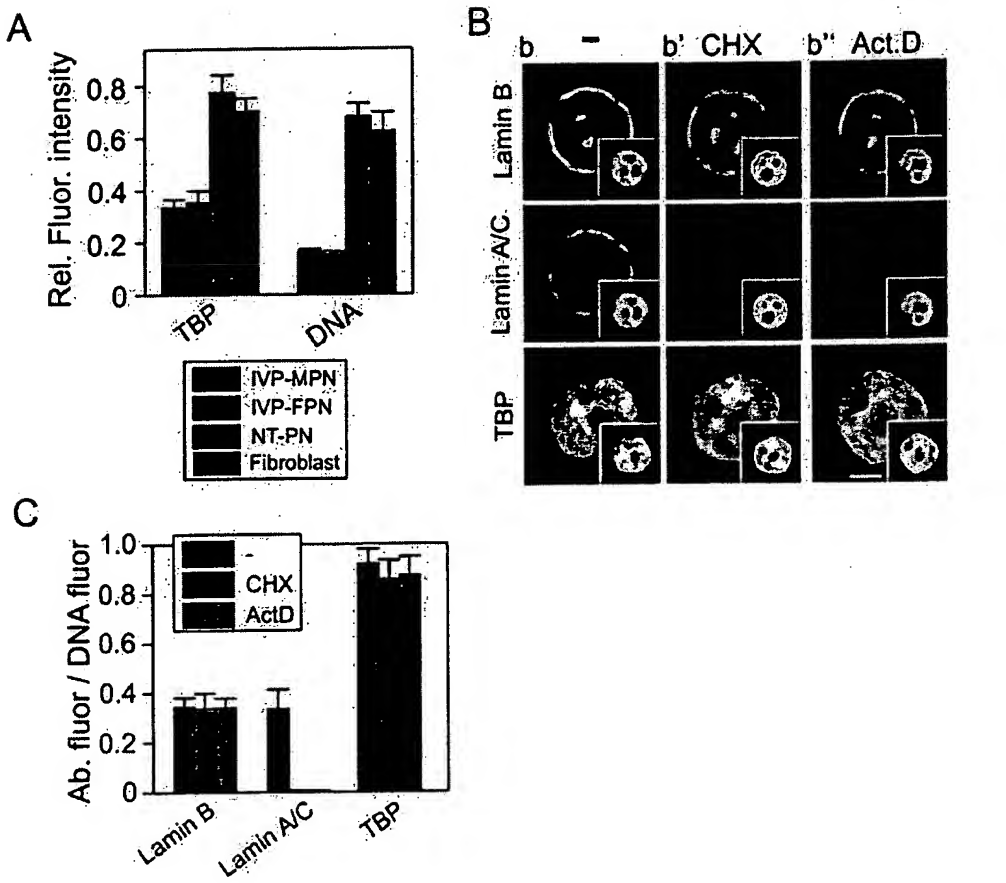

FIG. 2. Characterization of pronuclei in NT embryos. A) Immunolabeling intensity (mean ± SD) of TBP and DNA (Hoechst 33342) in pronuclear IVP and NT embryos and in fibroblasts after in situ extraction with 1% Triton X-100/1 mg/ml DNAse I/ 300 mM NaCl. Labeling intensity is expressed relative to that of nonextracted embryos (n = 30 embryos or cells per group). B) NT embryos were activated as (b) described in the text, or (b') in the presence of 10 μg/ml CHX or (b") 5 μg/ml ActD (n = 30 embryos/group in 2–3 replicates). Insets = DNA; bar = 10 μm. C) Ratio (mean ± SD) of immunolabeling intensity of indicated proteins over Hoechst 33342 (DNA) fluorescence in embryos activated as in B (n = 15–20 embryos/treatment/marker).

Within 3 h of transplantation of fibroblast nuclei into enucleated oocytes, donor chromosomes condensed and excluded both lamins A/C and B immunolabeling, whereas TBP remained associated with the chromosomes (Fig. 1C, CC). Fourteen hours after initiation of recipient oocytes activation, all NT embryos contained fully developed pronuclei with perinuclear lamin B labeling and TBP colocalized with DNA (Fig. 1C, NT-PN). However, in contrast to IVP embryos, 95–99% of NT embryos also displayed lamin A/C expression at the pronuclear stage (Fig. 1C), and expression persisted during early development (see below). Relative amounts of immunolabeled lamin B, lamin A/C, and TBP in pronuclei of NT and IVP embryos were quantified by measuring the ratio of secondary antibody fluorescence intensity to that of DNA (Hoechst 33342) to account for DNA content (haploid versus diploid) in the nuclei examined. Whereas relative amounts of lamin B were similar in pronuclei of NT and IVP embryos, relative amounts of lamin A/C and TBP were higher in NT pronuclei than in male (MPN) or female (FPN) pronuclei of IVP embryos (Fig. 1D).

TBP, DNA, and A-type Lamins in Pronuclei of NT Embryos

Higher amounts of TBP in NT pronuclei were associated with a greater resistance to in situ extraction with a combination of detergent (1% Triton X-100), nuclease (1 mg/ml DNAse I), and salt (0.3 M NaCl). Quantification of immunofluorescence labeling intensity in extracted embryos relative to that of nonextracted controls shows that ~35% of TBP remained unextracted in MPN or FPN of IVP embryos (Fig. 2A). However, TBP of NT pronuclei displayed strong resistance to extraction as in fibroblast nuclei (Fig. 2A). Similarly, DNA of NT pronuclei displayed a 4.5-fold increase in resistance to extraction under these conditions compared with pronuclei of IVP embryos (Fig. 2A, DNA). Altogether, the results suggest a more compact chromatin organization in pronuclei of NT embryos compared with IVP embryos.

To determine the origin of lamin B, lamin A/C, and TBP in pronuclei of NT embryos, recipient oocytes were activated in the presence of the RNA polymerase (Pol) II inhibitor ActD (5 μg/ml) or with the protein synthesis inhibitor CHX (10 μg/ml) [23]. Assembly of lamin A/C, lamin B, and TBP was examined by densitometric analysis of immunofluorescently labeled pronuclear embryos. Both inhibitors prevented pronuclear lamin A/C assembly (Fig. 2, B and C), suggesting that assembly of these somatic lamins in NT embryos resulted from transcription of the lamin A gene at the pronuclear stage. Lamin B assembly was not perturbed by CHX or ActD treatment (Fig. 2, B and C), suggesting that it was assembled from somatic lamins solubilized in the oocyte cytoplasm after NT and/or from a maternal pool of lamins. Similar amounts of TBP were detected in untreated embryos or after inhibition of RNA or protein synthesis (Fig. 2, B and C). Note that in IVP embryos, TBP remains undetectable (our unpublished data) until the pronuclear stage when it is upregulated (see Fig. 1A). Thus presumably TBP associated with condensed chromosomes in NT embryos represents a carry-over from the somatic nucleus, and TBP detected in NT pronuclei represents carry-over from the somatic nucleus in addition to any pronuclear stage embryonic production.

A Chromatin Transfer Strategy

In an attempt to alleviate defects identified in pronuclear NT embryos, we developed a procedure for directly ma-

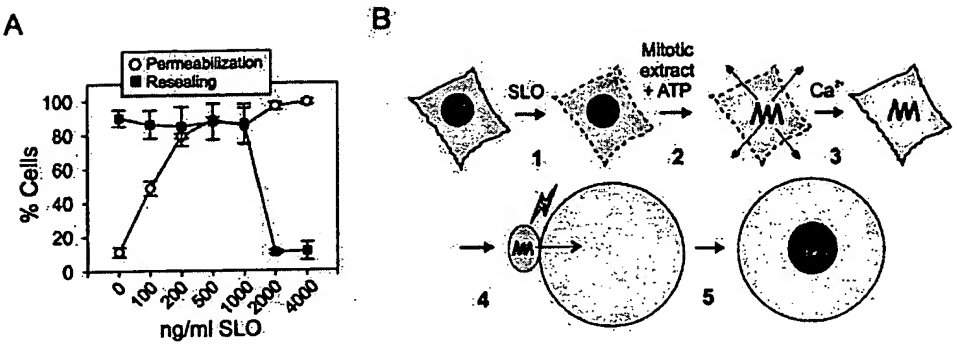

FIG. 3. Cloning by CT. A) Efficiency of SLO-mediated permeabilization and subsequent Ca^{2+}-mediated resealing of cultured bovine fetal fibroblasts, as determined by the proportion of cells taking up the membrane-impermeant DNA stain, propidium iodide. B) CT procedure: 1) Donor fibroblasts are reversibly permeabilized for 30 min with 500 ng/ml SLO; 2) permeabilized cells are washed and incubated for 45 min at 38°C in a mitotic extract containing an ATP-regenerating system to elicit chromosome condensation and promote removal of nuclear components (arrows); 3) extract is removed and cells resealed in culture with 2 mM $CaCl_2$ for 2 h; 4) resealed cells are fused to enucleated recipient oocytes; and 5) oocytes are activated as for NT to elicit pronuclear formation and development.

nipulating donor fibroblast nuclei in vitro prior to transfer into recipient oocytes. Fibroblasts were permeabilized with 500 ng/ml of the bacterial toxin Streptolysin O (SLO), as judged by uptake of the membrane-impermeant DNA stain, propidium iodide (Fig. 3A). Permeabilized fibroblasts were incubated in a mitotic extract for 45 min at 38°C to promote nuclear disassembly and removal of nuclear components (Fig. 3B; see below). The fibroblasts were recovered from the extract by sedimentation, washed, and cultured for 2 h with 2 mM $CaCl_2$ to reseal the plasma membrane [11] (Fig. 3, A and B). The resealed fibroblasts containing condensed chromosomes (as opposed to intact interphase nuclei; see below) were fused to recipient oocytes (Fig. 3B), and oocytes were activated with calcium ionophore/CHX/cytochalasin D as described for NT.

Breakdown of Fibroblast Nuclei in Mitotic Extract

The mitotic extract consisted of a 15 000 g supernatant from a lysate of mitotic MDBK cells and contained an ATP-regenerating system. The extract did not induce apoptosis, as judged by the absence of proteolysis of poly(ADP)ribosyl polymerase (PARP) and DNA fragmentation characteristic of apoptotic fibroblasts (Fig. 4A), and thus was suitable to promote remodeling of nuclei.

The extract elicited ATP-dependent condensation of chromosomes, disassembly of A- and B-type lamins from chromatin (as judged by immunolabeling of these lamins distributed throughout the cytoplasm), and removal of TBP from chromatin (Fig. 4B). These events were confirmed by immunoblotting analysis of condensed chromatin purified from the fibroblasts after recovery from the mitotic extract (Fig. 4C; compare lanes 1 and 3). In this experiment, the A-kinase anchoring protein AKAP95 [7] was used as a marker of a nuclear component that remains associated with the condensed chromosomes, as normally occurs at mitosis and upon chromosome condensation in vitro [7]. Histone H4 was used as a protein loading control in the gel (Fig. 4C). Disassembly of nuclear lamins and TBP from chromatin in mitotic extract was dependent on an ATP-regenerating system (Figs. 4B and 4C, lanes 3 and 4) and was reminiscent of that occurring in mitotic cells (Fig. 4C, lane 2). Furthermore, immunoblotting analysis of whole permeabilized fibroblasts (as apposed to isolated chromatin fractions) after exposure to the mitotic extract showed that a proportion of solubilized lamin A/C and all detectable TBP were eliminated from the cells and/or proteolyzed (Fig. 4C, lane 6). Finally, a control extract from interphase fibroblasts (Fig. 4C, lane 5) or cell lysis buffer alone (not shown), both containing an ATP-regenerating system, failed to promote nuclear disassembly, indicating that nuclear breakdown was specific for the mitotic extract. Permeabilized fibroblasts exposed to mitotic extract and subsequently resealed with 2 mM $CaCl_2$ in the culture medium could be cultured over several passages (data not shown). Thus membrane permeabilization, incubation of the permeabilized cells in the mitotic extract, and membrane resealing produced viable cells.

Characterization of Nuclei in Embryos Produced by Chromatin Transfer

Extract-treated and resealed fibroblasts were fused to recipient oocytes as efficiently (>70%) as nonpermeabilized control cells. The donor chromatin was in a condensed form at the time of introduction into the oocyte (Fig. 5A, CT). In contrast, chromatin of untreated control fibroblasts used for NT was still decondensed within 30 min of fusion (Fig. 5A, NT). Thus resealing of mitotic extract-treated fibroblasts with $CaCl_2$ prior to transfer into oocytes did not promote nuclear reformation in the donor cells. This observation was supported by the absence of a nuclear envelope around the condensed chromatin in CT embryos immediately after fusion, as judged by immunofluorescence analysis of lamina and inner nuclear membrane proteins (data not shown).

Immunolabeling of nuclear lamins and TBP in nuclei of CT and NT embryos, and immunolabeling intensity of these markers relative to DNA fluorescence intensity, are shown in Figure 5, B and C. Perinuclear lamin B labeling intensity was similar in CT and NT pronuclei. However, in contrast to NT embryos, lamin A/C was undetected in pronuclei and up to at least the 8–16-cell stage in CT embryos. CT pronuclei also displayed a fourfold reduction in TBP labeling compared with NT pronuclei (Fig. 5C). Pronuclear TBP concentration in the mouse has been shown to increase during progression through interphase [22]. However, as kinetics of pronuclear formation from donor chromatin condensed in the oocytes or from in vitro-condensed chromatin were similar in NT and CT embryos, respectively (unpublished data), it is unlikely, albeit not formally excluded, that enhanced TBP concentration in NT pronuclei was due to a more advanced cell cycle stage. Resistance of TBP to extraction with 1% Triton X-100, 1 mg/ml DNAse I, and 0.3

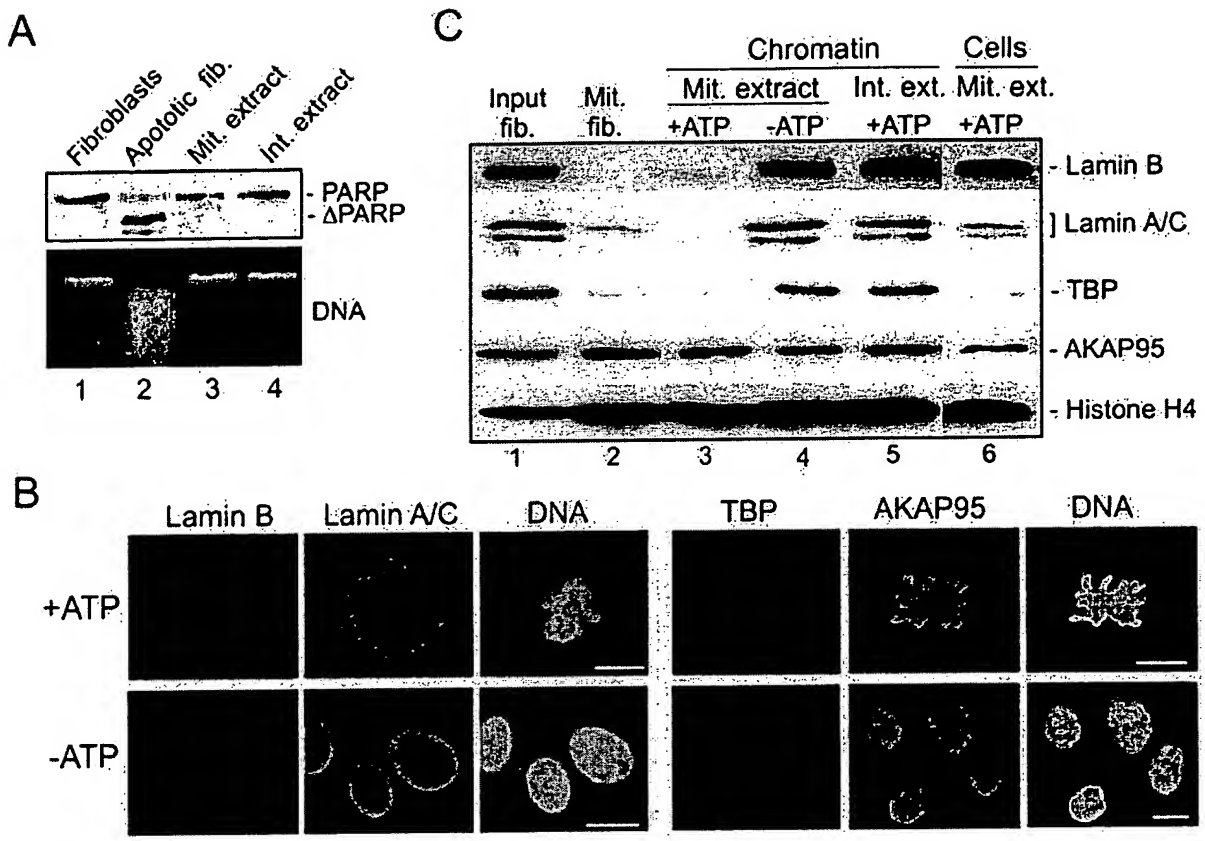

FIG. 4. Somatic nuclear breakdown in mitotic extract. A) Mitotic extract does not elicit apoptosis. Intact bovine fibroblasts (lane 1), fibroblasts induced into apoptosis with 5 μg/ml nocodazole for 20 h (lane 2), or SLO-permeabilized fibroblasts exposed to mitotic (lane 3) or interphase (lane 4) extract for 1 h were immunoblotted using anti-PARP antibodies (upper panel). ΔPARP indicates proteolyzed PARP. DNA degradation was evaluated by gel electrophoresis in 0.8% agarose and staining with ethidium bromide (lower panel). B) Immunofluorescence analysis of lamin B, lamin A/C, TBP, and AKAP95 after a 45-min incubation of permeabilized fibroblasts in mitotic extract with (+ATP) or without (−ATP) an ATP-regenerating system. Bars = 10 μm. Over 1000 cells analyzed in four replicates displayed consistent labeling as shown. C) Immunoblotting analysis interphase fibroblasts (lane 1); fibroblasts synchronized at mitosis with 1 μg/ml nocodazole (lane 2); chromatin isolated from permeabilized fibroblasts exposed to mitotic extract with or without the ATP-regenerating system (lanes 3, 4) or to interphase extract (lane 5); and whole permeabilized fibroblasts exposed to mitotic extract (lane 6). Blots were probed using antibodies to indicated proteins.

M NaCl was decreased by more than twofold (Fig. 5D), suggesting a weaker association of TBP with chromatin. Likewise, resistance of DNA to DNAse I was reduced nearly fourfold in CT pronuclei, suggesting that CT favors the establishment of a looser chromatin configuration in pronuclei (Fig. 5D).

Transfer into oocytes of fibroblasts synchronized in M-phase with nocodazole resulted in pronuclei with lamin A/C and TBP levels comparable with those of NT pronuclei (Fig. 5C, M-NT); highly DNAse I-resistant DNA; and TX-100-, DNAse I-, and NaCl-resistant TBP (Fig. 5D, M-NT). Donor metaphase chromosomes, however, were devoid of most detectable A- or B-type lamin or TBP labeling as shown biochemically (see Fig. 4C, lane 2). Absence of labeling was also evident at the chromatin condensation stage 3 h postfusion (not shown). Thus structural differences between NT and CT pronuclei detected earlier were not due to inconsistencies in cell cycle stage of the donor chromatin. We concluded that disassembly of fibroblast nuclei in mitotic extract followed by transfer of in vitro-condensed chromatin into oocytes enhanced morphological remodeling of the donor nuclei and alleviated defects detected in pronuclei of NT embryos.

Chromatin Transfer Produces Clones

CT resulted in development to term of cloned embryos (Fig. 6). In vitro development to blastocysts was similar for CT embryos (661/5880 cultured embryos, 11.2%) and NT embryos (1154/9362, 12.3%; $P > 0.1$). These figures represent data from six different bovine fetal fibroblast cell lines cloned both by CT and NT within an 18-month period. Notably, both CT and NT displayed a similar variation in the efficiency of development to blastocysts between cell lines (data not shown). Likewise, pregnancy rates (not shown) and proportions of calves born following transfer of blastocysts into recipient females were similar for NT and CT clones (11.2% [63/506 recipients] vs. 15.4% [42/273 recipients], respectively, $P > 0.1$). Furthermore, whereas the proportion of live calves at birth did not differ for NT and CT cloned animals (9% [46/506 recipients] vs. 10% [27/273 recipients], respectively, $P > 0.1$) across the six cell lines tested, the proportion of animals alive and healthy at 1 mo postpartum tended to be higher for CT (8.4%, 23/273 recipients) than for NT (5.1%, 26/506 recipients; $P = 0.07$; Fig. 6, A and B). Notably, however, CT did not obviously eliminate variations in development between cell lines cloned. Mean birth weight of CT and NT clones was not significantly different (data not shown). Altogether, our results indicate that CT produces live offspring and shows trends of improved viability of clones.

DISCUSSION

This is the first report of manipulation of a somatic nucleus in a cell extract as a step to enhance subsequent nu-

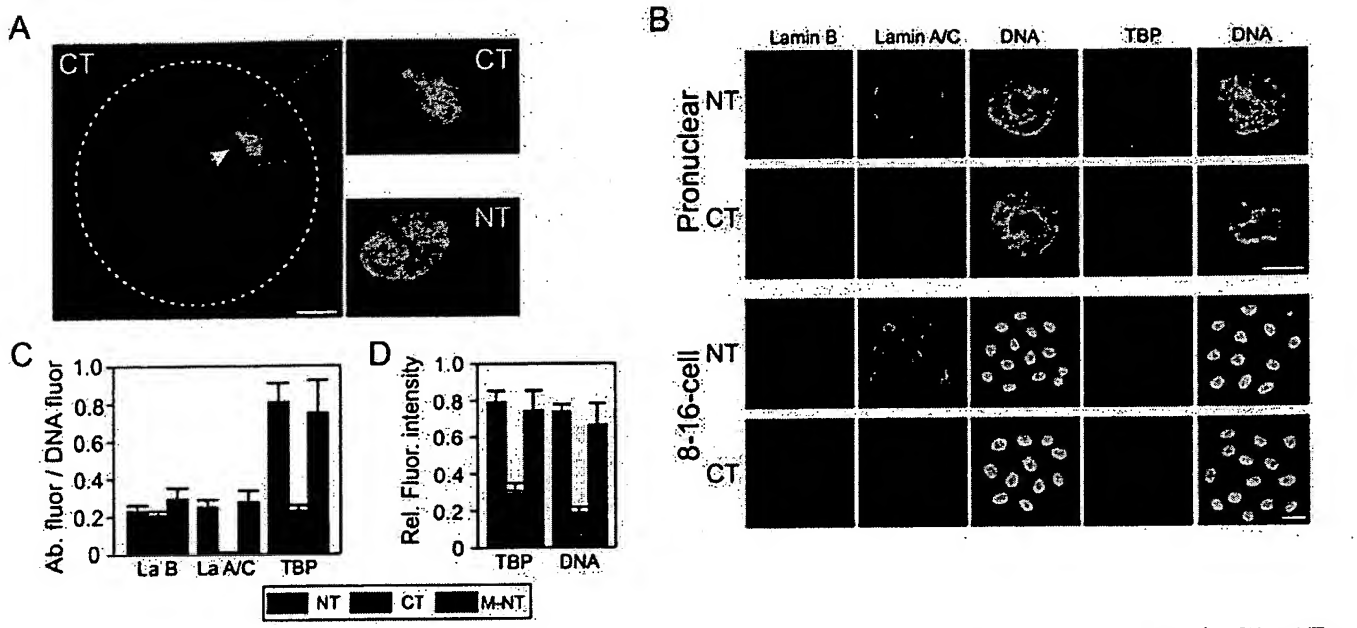

FIG. 5. Characterization of nuclei of CT embryos. A) Chromatin morphology (arrow) within 30 min of introduction into an oocyte by CT or NT, as indicated. Dotted line delineates the oocyte cytoplast. Panels on the right show enlargements of donor chromatin in a CT and NT embryo. Bar = 20 μm. B) Distribution of lamin B, lamin A/C, and TBP in pronuclear (upper two panels) and 8- to 16-cell-stage (lower two panels) embryos produced by NT or CT. Bars = 20 μm (n = 20–30 embryos analyzed per marker in three replicates). C) Ratio (mean ± SD) of immunolabeling intensity over Hoechst 33342 (DNA) fluorescence intensity for indicated protein in pronuclei of NT, CT, or M-NT embryos (n ~ 20 embryos/group/marker). D) Immunolabeling intensity (mean ± SD) of TBP and DNA (Hoechst 33342) in pronuclei of NT, CT, and M-NT embryos after extraction with 0.1% Triton X-100/1 mg/ml DNAse I/300 mM NaCl relative to that in nonextracted embryos (n = 15–30 embryos/group).

clear remodeling in the oocyte and efficiency of mammalian cloning. Permeabilization of the donor cell, induction of nuclear breakdown in a mitotic extract, and membrane resealing produces viable cells. The birth of cloned calves produced by CT supports this contention. The overall efficiency of producing cloned calves by CT appears similar to NT. Nevertheless, CT exhibits a trend toward enhanced survival of cloned calves at 1 mo postpartum. Moreover, in vitro breakdown of the somatic nucleus creates opportunities for directly accessing and manipulating the donor genome prior to introduction into the recipient oocyte. Remodeling of the somatic chromatin was demonstrated by induction of condensation of chromosomes in the mitotic extract. This clearly is distinct from a normal mitotic chromosome condensation, since condensation was elicited in fibroblasts from confluent cultures and therefore not in a G2 phase. One might anticipate possibilities for altering DNA methylation in donor cells prior to cloning to, tentatively, correct methylation defects in cloned animals [24–26]. Similarly, histone modifications might also be manipulated in vitro prior to cloning. Epigenetic manipulations of the donor genome might, speculatively, lead to enhanced development and health of clones.

We have identified several nuclear defects in NT embryos, including assembly of lamin A/C, enhanced pronuclear TBP content, and increased resistance of DNA to DNAse I. Our results complement recent observations in primate NT embryos resulting from somatic cell cloning, which display defects in mitotic spindle organization [27]. Abnormalities we observed may result from incomplete remodeling of the fibroblast nuclei and/or from misregulation of expression of differentiated cell-specific (e.g., lamin A) genes. Remodeling of nuclei in vitro and transplantation of condensed chromatin into oocytes alleviates these defects. Interestingly, however, transfer of M-phase cells into oocytes results in pronuclei resembling NT pronuclei with respect to lamin A/C and TBP expression and TBP anchoring. Thus chromosome condensation per se is not sufficient to rescue the defects at the pronuclear stage, suggesting that the cycle stage at which chromosome condensation takes

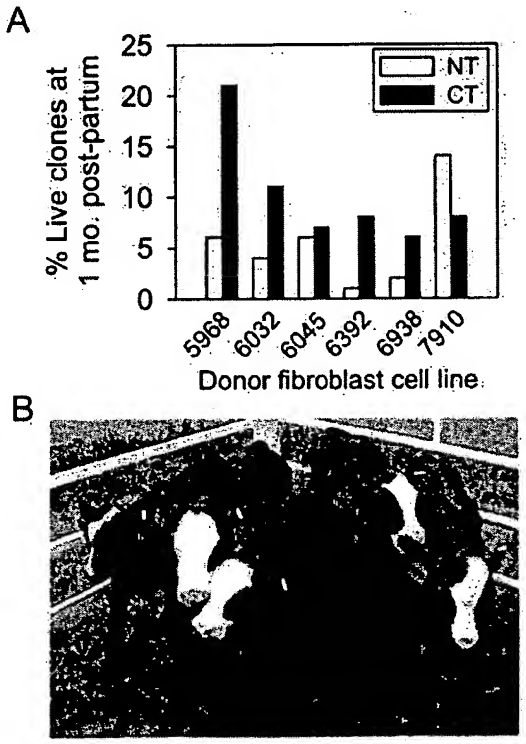

FIG. 6. Development of CT and NT clones. A) Percentages of live clones produced by NT and CT 1 mo after birth (percentage of recipient cows). See text for details. B) CT clones (Simintal × Angus cross identical females) at 5–6 wk of age.

place may be an important factor. A hypothesis is that in vitro remodeling through condensation of chromosomes during interphase alters the "memory" of chromatin organization in the somatic nuclei, which mere passage through a timely mitosis (i.e., after G2 phase) does not achieve [28]. This hypothesis remains to be tested.

Remodeling nuclei through CT increases DNA sensitivity to DNAse I and may promote the formation of transcriptionally active (or potentially active) chromatin. This may, in turn, facilitate expression of developmentally important genes. It will be interesting to identify and investigate the regulation of genes involved in placental development, maintenance of late pregnancy, and postnatal survival. CT also induces repression of lamin A gene expression in cloned embryos. In vitro and in vivo manipulations of nuclear lamina composition have shown that failure to assemble a correct set of lamins invariably leads to apoptosis [18]. Moreover, as lamins interact with DNA, chromatin, and the transcription machinery, proper lamina reconstitution is likely to be essential for normal nuclear function [15, 29] in cloned embryos.

Chromatin condensation at mitosis or in vitro is associated with the release of DNA-bound components such as chromatin remodeling enzymes [30], transcription. factors (such as TBP), or other potentially inhibitory somatic components. TBP removal from somatic nuclei was also induced in interphase *Xenopus* egg extracts as a result of ATP-dependent SWI-SNF-complex activity, but was not due to chromosome condensation [31]. It is not clear whether loss of TBP from fibroblast nuclei in our study results from chromosome condensation or SWI-SNF complex-related activities. In any event, TBP release from donor nuclei is more efficient under mitotic conditions (in vitro or during mitosis; Fig. 4) than under meiotic conditions (Fig. 1C). Resulting reduced TBP concentration in CT pronuclei, however, reflects dynamic transitions in nuclear structure and function [31]. In particular, removal of TBP from donor somatic chromatin may facilitate the repression or down-regulation of somatic-specific genes in CT embryos, which may impair development. An implication of removing factors from the donor nucleus is that loading of maternal components onto chromatin and subsequent remodeling into a physiological pronucleus may be facilitated.

In conclusion, we demonstrate that it is possible to remodel a somatic nucleus in a cell extract and produce live offspring. In vitro manipulation of nuclei for cloning or transdifferentiation purposes [10, 11] may constitute a useful tool for investigating the mechanisms of nuclear reprogramming. CT shows a trend toward improved viability of clones. Additional manipulation of the system might lead to further improvements in the efficiency of mammalian cloning.

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Cloned pigs produced by nuclear transfer from adult somatic cells

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Since the first report of live mammals produced by nuclear transfer from a cultured differentiated cell population in 1995 (ref. 1), successful development has been obtained in sheep[2,3], cattle[4], mice[5] and goats[6] using a variety of somatic cell types as nuclear donors. The methodology used for embryo reconstruction in each of these species is essentially similar: diploid donor nuclei have been transplanted into enucleated MII oocytes that are activated on, or after transfer. In sheep[2] and goat[6] pre-activated oocytes have also proved successful as cytoplast recipients. The reconstructed embryos are then cultured and selected embryos transferred to surrogate recipients for development to term. In pigs, nuclear transfer has been significantly less successful; a single piglet was reported after transfer of a blastomere nucleus from a four-cell embryo to an enucleated oocyte[7]; however, no live offspring were obtained in studies using somatic cells such as diploid or mitotic fetal fibroblasts as nuclear donors[8,9]. The development of embryos reconstructed by nuclear transfer is dependent upon a range of factors. Here we investigate some of these factors and report the successful production of cloned piglets from a cultured adult somatic cell population using a new nuclear transfer procedure.

To date, the efficiency of somatic cell nuclear transfer, when measured as development to term as a proportion of oocytes used, has been very low (1–2%)[10]. A variety of factors probably contribute to this inefficiency. These include laboratory to laboratory variation, oocyte source and quality, methods of embryo culture (which are more advanced in some species (such as cows) than others (such as pigs)), donor cell type, possible loss of somatic imprinting in the nuclei of the reconstructed embryo, failure to reprogram the transplanted nucleus adequately, and finally, the failure of artificial methods of activation to emulate reproducibly those crucial membrane-mediated events that accompany fertilization.

In the pig, there is the additional difficulty that several (> 4) good quality embryos are required to induce and maintain a pregnancy[11]. As fully developmentally competent embryos are rare in nuclear transfer procedures, there is every chance of squandering those good embryos unless very large numbers of reconstructed embryos are transferred back into recipients. Even if it were possible in the pig to select good quality blastocysts for transfer (after, for example, the use of a temporary recipient), most blastocysts formed from reconstructed embryos in other species are not competent to proceed to term[10]. The co-transfer of reconstructed embryos with 'helper', unmanipulated embryos, parthenotes or tetraploid embryos has been suggested as an aid to inducing and maintaining pregnancy. However, studies in mice after zygote pronuclear injection have suggested that the manipulated embryos are 'compromised' and selected against[12]. An alternative to the use of 'helper' embryos is the hormonal treatment of recipient sows to maintain pregnancy with low embryo numbers[13].

We cannot currently address all of the methodological problems, and, to improve our chances of success in pig nuclear

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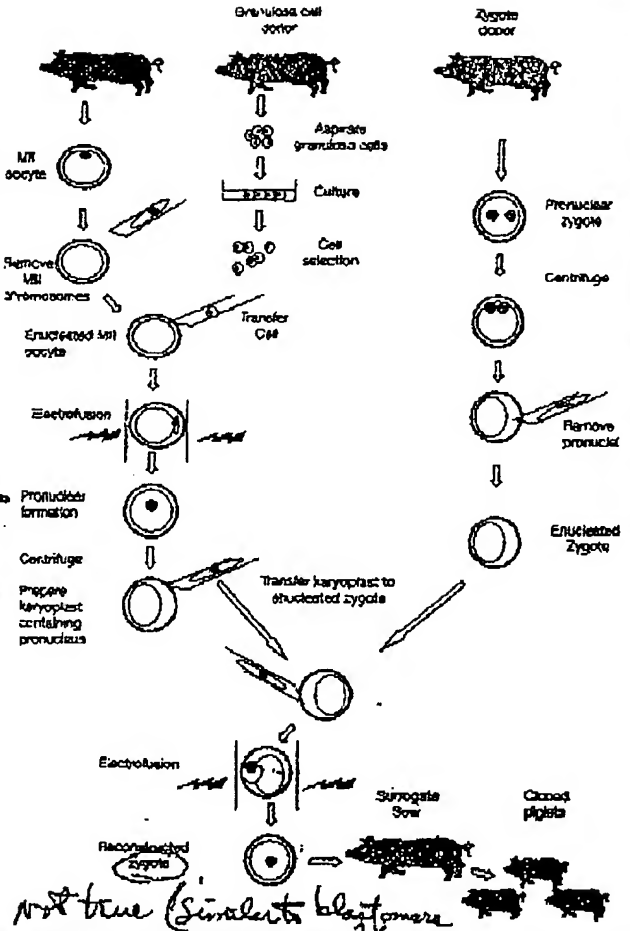

Figure 1 Representation of the double nuclear transfer procedure for the production of viable piglets using cultured adult somatic granulosa cells as nuclear donors. The outer circle in all the oocytes and embryos denotes the zona pellucida; the inner circle denotes the cell membrane.

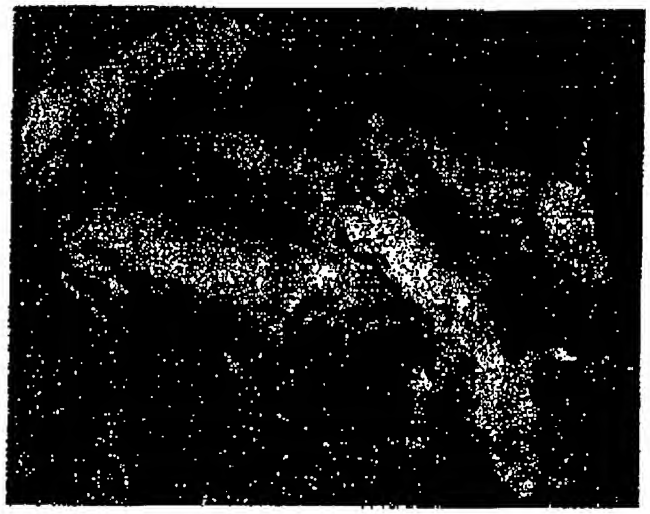

Figure 2 A litter of five live piglets derived by nuclear transfer using cultured adult granulosa cells as nuclear donors. A total of 72 reconstructed embryos were transferred to the surrogate sow.

transfer, we chose to focus on four areas: activation, choice of donor cell, embryo culture, and induction and maintenance of pregnancy.

In all species, when using MII oocytes as recipients, the method of activation is crucial for subsequent development. In the pig, although current activation protocols stimulate pronuclear formation, cleavage, and development to the blastocyst stage, both the frequency of development and the quality of the embryos produced are low[14]. A system that involves the use of fertilized zygotes as cytoplast recipients would bypass the inefficiencies of artificial activation procedures and might promote more successful development. The technique of pronuclear exchange between zygotes showed that the manipulations involved were compatible with development[15]; however, when donor nuclei from later developmental stages were transferred there was restricted development[16]. One explanation is that factors required for development, which are absent in the donor nuclei, are removed with the pronuclei. But if a pronucleus-like structure could be produced from the donor nucleus, this might prove a suitable nuclear donor for transfer. Such a system was described in mice by Kwon and Kono[17], who first fused mitotically arrested blastomere nuclei with enucleated MII oocytes. The reconstructed oocytes were subsequently activated in the presence of cytochalasin B, preventing polar body extrusion and resulting in the formation of two diploid pseudo-pronuclei. Each pseudo-pronucleus was then transferred into an enucleated, *in vivo* produced zygote, which was transferred into a surrogate recipient for development to term. Effectively, this latter procedure mimics pronuclear exchange and allows the

Table 1 Development of porcine embryos

	Double NT							Single NT		
Cell lipids	Poor	GR5	GR8	GR12	GR21Z	GR18	GR18	391	GR8	GR18
Cell treatment	CI	CI	CI	CI	CI	SS (74)	SS (51)	CI	CI	SS (51)
No. of oocytes	245	344	369	291	311	198	218	123	109	N/A
No. of attempted reconstructions day 1 (%)	190 (78)	217 (63)	207 (77)	226 (78)	221 (71)	122 (63)	192 (88)	94 (76)	83 (76)	N/A
No. of fused embryos day 1 (%)	124 (65)	153 (70)	186 (90)	97 (43)	163 (74)	90 (74)	162 (84)	87 (93)	61 (73)	N/A
No. of day-1 embryos with single pronucleus (%)	87 (70)	88 (57)	120 (64)	62 (64)	69 (42)	23 (26)	102 (63)	–	–	–
No. of reconstructed embryos day 2	74	57	105	67	55	22	45*	–	–	–
No. of fused embryos day 2 (%)	72 (97)	56 (98)	100 (95)	63 (97)	54 (98)	22 (100)	44 (98)	–	–	–
No. of embryos transferred to recipient	72	56	100	63	54	22	44	85	61	39†
Pregnancy (no. of fetuses observed)	+ve (5)	–ve	–ve	–ve	–ve	+ve (5)	+ve	–ve	–ve	–ve
No. of live births (%)	5 (7)	0	0	0	0	0	0	0	0	0

Development of porcine embryos reconstructed using a single or double nuclear transfer protocol with adult granulosa cells cultured to confluence (CI), or serum starved (SS) as nuclear donors.
* Insufficient zygotes to reconstruct day 1 embryos.
† Due to insufficient zygote numbers 39 day 1 reconstructed embryos were transferred to a single recipient.

formation of a final reconstructed one-cell embryo whose membrane has been activated during fertilization.

The use of cultured cell populations for the production of animals by nuclear transfer is now well documented in a number of species. We have considerable experience in the production of sheep and cattle from primary cell populations and genetically modified primary cell populations. Analysis of these studies has shown considerable variation in development between individual cell populations and at present has provided no definitive method for the identification of cell populations that are suitable for nuclear transfer. Factors that are thought to influence the suitability of a particular cell population include the effects of oxidative damage associated with cellular metabolism, genome instabilities and chromosomal pathologies. All of these factors may be influenced by the method of isolation and culture, and the number of population doublings in culture. On consideration of these factors and our previous observations, we chose to use granulosa cells as nuclear donors. Granulosa cells are suitable nuclear donors in cattle[16], and require the minimum of manipulations to establish in culture. Because of differences between cell populations, we initially decided to use a pool of cells isolated from a group of four donors. In later experiments, cell populations from individual animals were also examined. To minimize the culture period, early passage, never-frozen cells were used.

For embryo reconstruction, we attempted to minimize the potential inefficiencies at each step of the nuclear transfer procedure and adopted an approach that (1) uses *in vivo* derived material, (2) seeks to avoid artificial activation, and (3) minimizes the period of *in vitro* culture of manipulated embryos. To do this we used a two-stage nuclear transfer procedure modified from Kwon and Kono[17] (Fig. 1). In the first stage, donor cells were fused to *in vivo* derived, enucleated MII oocytes obtained from superovulated crossbred gilts. The pseudo-pronucleus formed in the first nuclear transfer embryo was then subsequently transplanted into an *in vivo* produced, enucleated zygote (second nuclear transfer embryo). The second nuclear transfer reconstructed embryo was transferred to the oviduct of a synchronized sow within 2 h of fusion. Because of the expected low developmental rate, we transferred up to 100 reconstructed embryos to a single recipient. Each recipient was treated with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) to maintain pregnancy[18] in the event that fewer than four reconstructed embryos were viable at implantation.

Coordination of the cell-cycle stages of the recipient cytoplasm and the donor nucleus are essential for maintaining correct ploidy and preventing DNA damage in nuclear transfer reconstructed embryos[19]. Various combinations of donor and recipient cell-cycle stages can prevent DNA damage and uncoordinated DNA replication, and result in formation of a pseudo-pronucleus. It has been suggested that the use of MII oocytes may improve 'reprogramming' of the donor genetic material owing to the occurrence of nuclear envelope breakdown and premature chromosome condensation, thus exposing the donor chromatin to maternally derived oocyte factors involved in early development. To take advantage of this here, we used MII oocytes as cytoplast recipients for the first nuclear transfer embryo reconstruction. To maintain ploidy in this situation, we chose diploid donor nuclei as nuclear donors.

Previous studies have suggested that diploid cells arrested in the G0 phase of the cell cycle may be beneficial[1]. Using flow cytometry, we examined the cell-cycle distribution of porcine granulosa cells under three different culture conditions: sub-confluent actively growing, 100% confluent, and cells starved of serum for 48 hours (see Figure in Supplementary Information). After serum starvation, the population contained a large proportion (7.2%) of cells with a DNA content lower than that consistent with a diploid cell (termed sub-G1). In contrast, in the population synchronized by contact inhibition, 90.3% of the cells had a diploid DNA content (G1/G0) and there were fewer sub-G1 cells (1.6%). We analysed DNA synthesis in serum-starved and contact-inhibited cell populations by 5-bromo-2′-deoxyuridine (BrdU) incorporation. These experiments revealed that 45% of the contact inhibited cell population compared with 0% of the serum-starved population incorporated BrdU. An analysis of BrdU incorporation after an additional 24 h of contact inhibition revealed that the fraction of BrdU-positive cells was reduced to 5%. These observations suggest that the diploid cells in the contact-inhibited granulosa cell population used as nuclear donors for embryo reconstruction contained a mixture of cell-cycle-arrested diploid cells (G1/G0) and unarrested diploid cells (G1), which were able to undergo a further round of DNA synthesis. In

Table 2 Microsatellite analysis of pigs and cell donors

Loci	S0059	S0070	S0122	S0226	SW24	SW72	SW840	SW936	TNFB
Samples									
PGR1	152	275	178	178	109	102	129	95	161
	152	295	182	198	111	110	129	97	164
PGR2	148	275	180	178	103	110	129	97	158
	154	275	182	198	111	112	129	111	165
PGR3	148	275	180	178	103	110	125	97	N/A
	152	275	182	198	109	112	125	111	
PGR4	152	275	178	198	103	102	129	97	158
	152	295	182	198	111	110	129	109	161
NTP1	152	275	178	178	103	102	129	95	161
	152	295	182	198	111	110	129	97	164
NTP2	152	275	178	178	103	102	129	95	161
	152	295	182	198	111	110	129	97	164
NTP3	152	275	178	178	103	102	129	95	161
	152	295	182	198	111	110	129	97	164
NTP4	152	275	178	198	103	102	129	97	158
	152	295	182	198	111	110	129	109	161
NTP5	152	275	178	198	103	102	129	97	158
	152	295	182	198	111	110	129	109	161
Recipient (54B)	152	275	178	182	111	102	N/A	103	158
	152	295	182	198	96	102		109	161
Boar	134	265	178	180	115	102	129	97	164
	156	273	182	180	115	112	129	109	165

Microsatellite analysis was performed on genomic DNA from the four individual populations of granulosa cells (PGR1, PGR2, PGR3, PGR4), the piglets (NTP1–5), the surrogate sow (54B) and the boar responsible for inseminating the zygote donors. Primers corresponding to nine polymorphic loci were used. Two numbers are shown for each sample at each locus, which represent the PCR product size for each of the two alleles at that particular locus.

contrast, when the serum-starved populations were used as nuclear donors most the diploid cells were cell-cycle arrested (G1/G0).

Production of the first nuclear transfer embryos requires activation of the *in vivo* derived oocytes. Activation experiments carried out in control oocytes showed that electrical stimulation applied between 51.5 and 60 h after hCG administration, promoted similar cleavage and development to blastocyst (see Table in Supplementary Information). For embryo reconstruction, MII oocytes were collected 46–54 h after hCG and the first nuclear tranfer embryo reconstruction was carried out between 50 and 58 h after hCG. Reconstructed embryos were cultured overnight in NCSU-23 medium[22]; we then checked them for the presence of a pronucleus and used them for the second nuclear transfer embryo reconstruction. The development of single nuclear transfer and double nuclear transfer embryos reconstructed from contact-inhibited and serum-starved granulosa populations were compared (see Table 1). In total, 185 single nuclear transfer embryos were transferred to 3 recipient sows and 401 double nuclear transfer embryos to 7 recipients. Two recipients of the double nuclear transfer embryos became pregnant as determined by ultrasound visualization of fetuses at day 35 of gestation. One of these maintained the pregnancy to term, and five piglets (Fig. 2) were delivered by Caesarean section on day 116 of gestation. The average birth weight of the piglets was 2.72 lb (range 2.28–3.08 lb); this is about 25% lower than that observed in the same population of pigs under natural mating conditions (average litter size average 10.9, average birth weight 3.6 lb, range 3.3–3.9 lb).

The five piglets were produced from a pooled population of cells derived from four animals. We carried out microsatellite analysis of genomic DNA from the various samples (Table 2). The comparison of the pattern of alleles in the piglets with that of the granulosa cell populations indicated that three of the nuclear transfer piglets (NTP1, NTP2 and NTP3) were derived from the porcine granulosa (PGR) 1/cell line, as there was 100% identity at all nine microsatellite markers. The other 2 nuclear transfer piglets (NTP4 and NTP5) showed perfect identity with the genotype of the PGR4 cell population. All five of the nuclear transfer piglets were significantly different from the surrogate mother (54B). Some of the loci (S0059, S0070, S0122 and TNFB) were not highly polymorphic indicating a degree of homogeneity or inbreeding within the population of pigs used in these studies (all of which come from the same commercial supplier).

We think that the principal reasons for the success of this modified nuclear transfer procedure in pigs is its lack of reliance on current artificial activation protocols and *in vitro* culture techniques. Although elaborate, the double nuclear transfer does not add another major inefficiency (the second step fusion is very efficient). Direct transfer of a somatic nucleus to an enucleated zygote will not work because (in addition to reprogramming difficulties) of the loss of important factors sequestered within the removed pronuclei. The cell population used successfully as nuclear donors in these experiments were not quiesced by serum starvation. Cell-cycle analysis showed that most cells in control cultures had a diploid DNA content, and a high percentage were able to undergo a further round of DNA synthesis suggesting that most cells in the population were in the G1 phase of the cell cycle and not arrested in G0. All five of the pigs, now three months old, are extremely healthy, in contrast to the (usual) 50% postnatal loss of nuclear transfer animals[10]. It is tempting then to speculate that this modified method may have general utility in other species, even those where single nuclear transfer has been shown to work.

The successful development of nuclear transfer in pigs opens the door for the application of gene-targeting technology, thus allowing for very precise genetic modifications, including gene knockouts. We have recently reported gene targeting in cultured ovine somatic cells and the successful development to term of offspring produced by nuclear transfer using these cells[20]. In pigs, a gene of great interest for the application of knockout technology is that for α-1,3-galactosyl transferase (α-1,3-GT)—the enzyme responsible for adding the xenogeneic sugar, galactose α-1,3-galactose, to the surface of porcine cells. This gene is inactive in certain monkeys and humans, and their blood contains anti-gal antibodies, which trigger (in monkeys) early rejection of transplanted organs[21]. We have achieved targeted disruption of the α-1,3-GT gene in primary porcine cells (unpublished data) and this will allow the production of α-1,3-GT-deficient pigs, whose organs should show improved resistance to rejection. Overcoming antibody-mediated rejection is the first critical step in improving xenograft survival, towards the ultimate goal of providing an unlimited supply of compatible pig organs for human transplantation. □

Methods

Modified NCSU-23 medium

The published NCSU-23 medium[22] was modified for use as a phosphate-buffered benchtop medium without $NaHCO_3$. Physiological pH phosphate buffer is made using a 3:1 molar ratio of dibasic to monobasic phosphate anions. These changes induced alterations in the Na and K concentrations, which were corrected by adjusting the NaCl and KCl concentrations to maintain osmolarity and Na/K ratio (all chemicals purchased from Sigma unless otherwise noted).

Superovulation of donor gilts for collection of oocytes and zygotes

Crossbred gilts (280–320 lbs) were synchronized by oral administration of 18–20 mg Regu-Mate (Altrenogest, Hoechst) mixed into the feed. Regu-Mate was fed for 5–14 d using a scheme dependent on the stage of the oestrous cycle. Estrumate (250 μg, Bayer) was administered intramuscularly (i.m.) on the last day of the Regu-Mate treatment. Superovulation was induced with a single i.m. injection of 1,500 IU of PMSG (Diosynth) 15–17 h after the last Regu-Mate feeding. One thousand units of hCG (Intervet America) were administered i.m. 82 h after the PMSG injection.

We collected oocytes 46–54 h after the hCG injection by reverse flush of the oviducts using pre-warmed Dulbecco's phosphate buffered saline (PBS) containing bovine serum albumin (BSA; 4 g l^{-1}). For the collection of zygotes, 24–36 h after the hCG injection the gilts were either artificially inseminated or bred naturally. We flushed zygotes from the oviduct 52–54 h after the hCG injection using PBS containing BSA (4 g l^{-1}).

Isolation and culture of porcine granulosa cells

Follicular fluid was aspirated from 2–8-mm diameter follicles of superovulated crossbred gilts (Large White (1/2), Landrace (1/4), White Duroc (1/4)), 7–8 months old, 280–320 lbs, 48–51 h post hCG injection. Granulosa cells were collected by centrifugation at 1,040 *g* for 10 min, re-suspended in DMEM (Gibco), containing 10% fetal calf serum (FCS; Summit Biotech), 0.1 mM non-essential amino acids (NEAA Gibco), 2 ng ml^{-1} basic fibroblast growth factor (bFGF) (Beckton Dickinson) and 4 μl ml^{-1} Gentamycin (Sigma). Cells were expanded for several days and then cryo-preserved.

For nuclear transfer, we plated the granulosa cells at $1–3 \times 10^4$ cells per 35 mm dish in DMEM medium supplemented with NEAA (0.1 mM), bFGF (2 ng ml^{-1}) and 10% FCS, and cultured them to 100% confluency at 37 °C. For experiments where serum starvation was evaluated, cells were starved of serum for 48–72 h in DMEM containing 0.5% FCS. We collected cells by trypsinization and stored them in suspension in modified NCSU-23 phosphate medium at 38.5 °C for 20–120 min, before use as nuclear donors.

Activation of oocytes

Activation of control oocytes was achieved by application of two 1.0 kV cm^{-1} DC electric pulses for 60 μs each at an interval of 5 s in activation medium (0.3 M D-sorbitol supplemented with 0.1 mM $Mg SO_4$ and 0.05 mM $CaCl_2$ in H_2O).

Reconstruction of first nuclear transfer embryo

Recovered oocytes were washed in PBS containing 4 g l^{-1} BSA at 38 °C, and transferred to calcium-free phosphate-buffered NCSU-23 medium at 38 °C for transport to the laboratory. For enucleation, we incubated the oocytes in calcium-free phosphate-buffered NCSU-23 medium containing 5 μg ml^{-1} cytochalasin B (Sigma) and 7.3 μg ml^{-1} Hoechst 33342 (Sigma) at 38 °C for 20 min. A small amount of cytoplasm from directly beneath the first polar body was then aspirated using an 18-μM glass pipette (Humagen, Charlottesville, Virginia). We exposed the aspirated karyoplast to ultraviolet light to confirm the presence of a metaphase plate. A single granulosa cell was placed below the zona pellucida in contact with each enucleated oocyte. The couplet was transferred to a fusion chamber (model no. BT-453, BTX Inc., San Diego) containing 700 μl of 0.3 M mannitol, 0.1 mM $MgSO_4$ and 0.1 mM $CaCl_2$ in deionized water. Fusion and activation were induced by application of an AC pulse of 5 V for 5 s followed by two DC pulses of 2.5 kV cm^{-1} for 60 μs using an ECM2001 Electrocell Manipulator (BTX Inc., San Diego). Couplets were then washed in bicarbonate buffered NCSU-23 medium, and incubated in this medium for 0.5–1 h at 38.5 °C in a humidified atmosphere consisting of 5% CO_2 in air. We checked couplets for fusion at ×300 magnification using an inverted microscope. Fused embryos were given a second activation stimulus of two successive DC pulses of 1.2 kV cm^{-1} for 60 μs each, and cultured overnight in NCSU medium[22].

Reconstruction of second nuclear transfer embryo

Zygotes were centrifuged at 14,900 *g* for 15 min in a Biofuge 13 centrifuge and then incubated in phosphate buffered NCSU-23 medium containing 5.0 μg ml^{-1} cytochalasin B (Sigma) at 38 °C for 20 min. Zygotes containing two pronuclei were enucleated using a 25–35-μm glass pipette by aspirating a membrane bound karyoplast containing both pronuclei (and the second polar body if present). Karyoplasts containing the pseudo-pronucleus were prepared from the day 1 nuclear transfer embryos as described for zygote enucleation with the modification that a 30–45-μm enucleation pipette was used for manipulation. A single karyoplast was placed into the perivitelline space of each enucleated zygote. Fusion was induced by application of an AC pulse of 5 V for 5 s followed by two DC pulses of 1.2 kV cm^{-1} for 60 μs. Couplets were then washed and cultured in NCSU-23 medium for 0.5–1 h at 38.5 °C in a humidified atmosphere of 5% CO_2. We transferred fused couplets as soon as possible to the oviduct of an oestrus-synchronized recipient gilt.

Treatment of recipient sows

Pregnancy was maintained by using a combination of PMSG and hCG. PMSG (1,000 IU) was injected i.m. on day 10 of the oestrous cycle (day 1 being the day of oestrus), hCG was injected i.m. 3–3.5 d later (day 13 of the cycle)[19].

Microsatellite analysis

DNA (25 ng μl^{-1}) from each of the five piglets (24-h tail samples), the four granulosa cell lines mixed to make pool 1 (PGR1, PGR2, PGR3 and PGR4), the recipient sow (54B) and a boar, 'Ranger', that was used for artificial insemination purposes, were sent in individually coded vials to Celera-AgGEN (Davis, California), a company that specializes in parentage verification for swine. The microsatellite analysis consists of a multiplexed set of nine polymorphic porcine loci, each of which consists of different multimers of short tandem repeats (dinucleotides). The polymorphic loci are designated: S0059, S0070, S0122, S0226, SW24, SW72, SW840, SW936 and TNFB (PCR primer sequence information is proprietary to Celera-AgGEN). This multiplex set contains nine different PCR primer pairs, amplified in two PCR reactions, a five-plex and a four-plex. Ten nanograms of template DNA was amplified in each multiplex PCR. The forward primers were labelled on the 5′ end with a fluorescent dye (either FAM, JOE or TAMRA). The entire battery for each DNA sample was loaded in a single lane and co-electrophoresed with the internal size standard GeneScan 350 Rox. All multiplexing and loci evaluations were performed on an ABI PRISM 377 DNA Sequencer and analysed with Genotyper 2.0 software.

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Infection by porcine endogenous retrovirus after islet xenotransplantation in SCID mice

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Animal donors such as pigs could provide an alternative source of organs for transplantation. However, the promise of xenotransplantation is offset by the possible public health risk of a cross-species infection[1,2]. All pigs contain several copies of porcine endogenous retroviruses (PERV)[3,4], and at least three variants of PERV can infect human cell lines *in vitro* in co-culture, infectivity and pseudotyping experiments[3,5–7]. Thus, if xenotransplantation of pig tissues results in PERV viral replication, there is a risk of spreading and adaptation of this retrovirus to the human host. C-type retroviruses related to PERV are associated with malignancies of haematopoietic lineage cells in their natural hosts[8]. Here we show that pig pancreatic islets produce PERV and can infect human cells in culture. After transplantation into NOD/SCID (non-obese diabetic, severe combined immunodeficiency) mice, we detect ongoing viral expression and several tissue compartments become infected. This is the first evidence that PERV is transcriptionally active and infectious cross-species *in vivo* after transplantation of pig tissues. These results show that a concern for PERV infection risk associated with pig islet xenotransplantation in immunosuppressed human patients may be justified.

Juvenile-onset diabetes mellitus is a major health problem and exogenous insulin therapy is only partially successful in preventing its many complications. Although islet transplantation holds great promise for a cure, the number of potential human pancreas donors are extremely unlikely to provide enough islet tissue to treat the millions of patients worldwide. The xenotransplantation of pig

Production of cloned pigs from in vitro systems

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Here we describe a procedure for cloning pigs by the use of in vitro culture systems. Four healthy male piglets from two litters were born following nuclear transfer of cultured somatic cells and subsequent embryo transfer. The initiation of five additional pregnancies demonstrates the reproducibility of this procedure. Its important features include extended in vitro culture of fetal cells preceding nuclear transfer, as well as in vitro maturation and activation of oocytes and in vitro embryo culture. The cell culture and nuclear transfer techniques described here should allow the use of genetic modification procedures to produce tissues and organs from cloned pigs with reduced immunogenicity for use in xenotransplantation.

Keywords: Nuclear transfer, oocyte maturation, oocyte activation, embryo culture, porcine, in vitro fertilization

We report here the successful cloning of pigs by somatic cell nuclear transfer (NT). Despite the demonstrated success of cloning using somatic cells from several other animal species including sheep[1,2], cattle[3,4], goats[5], and mice[6], porcine NT has proved to be more challenging[7–11]. Indeed, although two groups have recently reported the birth of cloned piglets[12,13], neither group used the simple method of fusing nuclear donor cells into in vitro matured, enucleated oocytes to produce these clones. We have systematically optimized each step in the NT procedure, including the source of oocytes and their maturation in vitro, the culture of donor cells, the activation of oocytes following NT, and the in vitro culture of embryos and their transfer to recipient gilts. The result is a reproducible methodology that should enable strategies to genetically modify pigs for xenotransplantation, the production of pharmaceutical proteins, and the enhancement of pig breeding programs.

The shortage of human organs for allotransplantation has motivated a search for alternative sources. Xenotransplantation of pig organs is an attractive option because of the compatible size, physiology, and potentially large supply[14]. The major hindrance to using pig organs is immunological incompatibility[15]. In particular, transplantation from pigs to humans results in hyperacute rejection (HAR), which is mediated by preformed xenoreactive antibodies with specificity for the Gal-α-1,3-Gal epitope on the surface of pig endothelial cells[16].

Ideally, this epitope would be completely ablated using gene targeting methods to inactivate the gene encoding α-1,3-galactosyltransferase. The demonstration that gene targeting in embryonic stem (ES) cells could be used to manipulate the germline in mice was first reported in 1989[17]. However, strategies used to generate targeted knockout of mouse genes have failed in pigs[18,19]. Somatic cell NT in pigs is the most promising technology to achieve the targeted knockout of the α-1,3-galactosyltransferase gene, in that donor cells can be genetically modified before NT using existing technologies[20].

The major limitation to the genetic manipulation of donor cells is the length of time transfected cells must be cultured to allow selection, colony growth, and genetic testing preceding NT. Certainly the ability of these cells to undergo a second round of gene targeting to remove a second allele could be limited. However, genetically modified donor cells can be used to produce a cloned fetus, providing cells that can be used for additional rounds of targeting to remove a second allele, or to target additional genes. This approach should be successful because it has been shown that senescent bovine cells can be "rejuvenated" by NT and that cloned fetal cells are totipotent[21] (ref. 21 and unpublished data).

Results and discussion

Nuclear donor cells used to produce the two litters of cloned piglets (Fig. 1) were derived from 47- and 51-day porcine fetuses, respectively. The cells were cultured for 8 or 22 days and passaged 0 or 2 times prior to NT (Table 1) and had an elongated morphology in culture (Fig. 2). Five additional pregnancies have been initiated using fetal porcine cells that were cultured from 9 to 87 days and passaged from 0 to 7 times (Table 1). Fetal porcine cells have been maintained in culture greater than 200 days and passaged at least 8 times without visible senescence (not shown). Cells were used in nuclear transfer 0–4 days after reaching confluence. This contrasts with the two recent reports of pig cloning where the cells used in NT were either serum starved adult granulosa cells[12] or fetal fibroblasts cultured for 16 days after achieving confluence[13]. The assumption in these reports was that cells used in NT had to be in the G1/G0 cell cycle phase for successful cloning. However, the lack of intervention here to synchronize the cells does not support this assumption.

Using the methods described here, the rate of pregnancy initiation was 30% (seven initiations, including the two births, out of 23 recipients that received NT embryos). This rate of pregnancy initiation was similar to that reported by Polejaeva *et al.* (29%)[13], who used a double nuclear transfer method, but could not be compared to the report by Onishi *et al.*[12] because ultrasound verification of pregnancy was not performed. In this second report, donor nuclei were directly injected into enucleated oocytes in contrast to the method used here where the donor cells were fused with the enucleated oocyte. Transfer of the entire donor cell cytoplasm into the enucleated oocyte by fusion to form a cytoplasmic hybrid apparently did not interfere with the reprogramming of the donor nuclei.

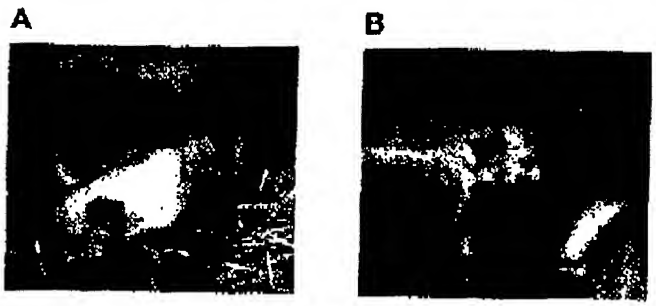

Figure 1. Nuclear transfer piglets from two litters. The piglets were photographed nine days (A) and one day (B) after birth.

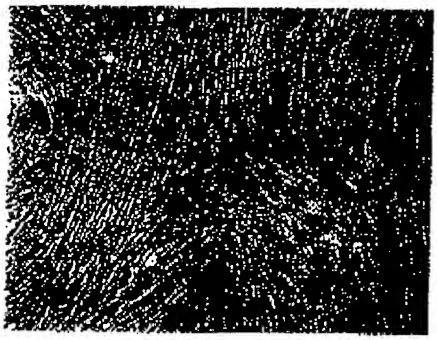

Figure 2. Porcine donor cells. Body cells from a 47-day fetus were cultured to confluence preceding nuclear transfer. These cells were used to produce the first litter of cloned piglets.

Four of the pregnancies listed in Table 1 were derived from donor cells transfected with a DNA construct that included a *neo* cassette to confer resistance to neomycin derivatives. One of the pregnancies (no. 3 in Table 1) from transfected cells terminated at 33 days into gestation. A fetus was recovered for transgene analysis however it did not contain the *neo* gene, most likely because the population of transfected cells used in NT was a mixture of nontransgenic and transgenic cells.

Parentage analysis was performed on the NT piglets and the surrogate recipients to confirm identity to the donor cell line used for NT. DNA was extracted from tissue samples acquired from each newborn piglet (ear punch) and the recipients (blood). Eleven porcine DNA microsatellite (MS) markers were used to confirm the genetic identity of the cloned piglets to cells used in NT. Results of the MS marker analysis verified that the donor cell lines were the source of genetic material used to produce the newborn piglets (Table 2; Fig. 3) and the recovered fetus (not shown).

Oocytes obtained from sows (animals that had given birth) and gilts (animals that had not given birth) were treated identically during in vitro maturation and subsequent embryo culture. When sow oocytes were used for NT, 8% (15/192) of the cytoplasmic hybrids developed to blastocyst compared to only 4% (11/258) when using gilt oocytes. Similarly, when sow oocytes were used for in vitro fertilization (IVF), 22% (86/384) of the inseminated oocytes developed to blastocyst compared to 14% (80/584) when using gilt oocytes. In addition, litter size was larger when oocytes were derived from sows (9.0) rather than from gilts (5.0), although IVF embryos derived from both sow and gilt oocytes produced a 53% pregnancy initiation rate (10/19 each). A plausible explanation for the higher rate of development using sow oocytes is that a greater percentage of sow oocytes are amenable to in vitro maturation compared to gilt oocytes when using the methods described here. Although sows are reproductively mature preceding slaughter, gilts vary in maturity. In fact, reduced fertility and litter size has been described for gilts[12]. A

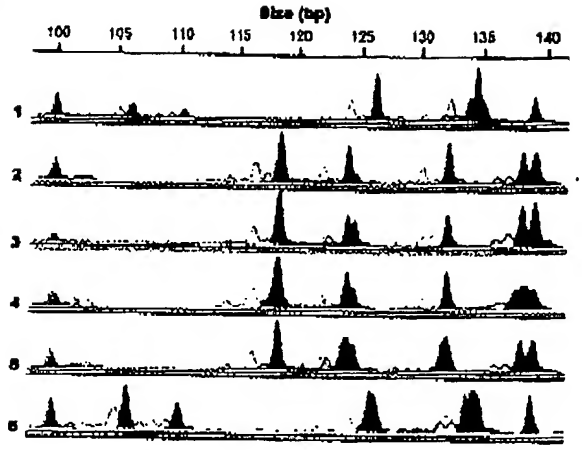

Figure 3. Multiplex electropherogram of three microsatellite markers. The six electropherograms represent analysis of tissue samples from the first litter of cloned piglets: Recipient sow (1), donor cell line (2), cloned piglet one (3), cloned piglet two (4), tissue source of donor cell line (5), and a second sample of the recipient sow (6). The green peaks represent alleles of the SW-268 locus, the blue peaks represent the SW-66 locus, and the black peaks represent the SW-1510 locus. The red peaks show the internal size standards; the calculated sizes (in base pairs) are displayed at the top of the figure.

Table 1. Porcine births/pregnancies from nuclear transfer derived embryos

No.	1	2	3	4	5	6	7
Originating fetal Age (days)	47	51	41	41	56	51	51
Cell source	Body	Genital ridge	Body[a]	Body[a]	Genital ridge	Body[a]	Body[a]
Culture age at nuclear transfer (days)	22	8	73	87	9	84	86
Passage number	2	0	6	7	0	4	4
No. nuclear transfers	143	340	198	175	192	135	186
Time in embryo culture (h)	8	76	76	72	76	76	76
No. of embryos transferred into recipient	143	164	121	116	120	115	123
Pregnancy status	Litter 1 July[b]	Litter 2 Sept.[c]	Abort (33 days)	Due Oct.	Abort (40 days)	Due Nov.	Due Nov.

[a]These cell lines were transfected with a DNA construct containing *neo*.
[b]Two male piglets were born alive by vaginal delivery and weighed 2 and 3 pounds, respectively.
[c]Two male piglets and a mummified fetus were born by vaginal delivery. The live piglets weighed 2.2 and 3.5 pounds, respectively.

Table 2. DNA parentage analysis[a]

Marker	Dye name	PCR annealing temp.	Recipient 1 genotypes	Cell line 1 genotypes	Litter 1 genotypes	Recipient 2 genotypes	Cell line 2 genotypes	Litter 2 genotypes
SW1332	HEX	65.0	98/114	96	96	88/96	88/96	88/96
SWR136	TET	64.2	-	-	-	193/203	214/220	214/220
SWR1120	FAM	63.3	176	168/176	168/176	164/176	164/176	164/176
SWR308	TET	62.2	142/157	127/142	127/142	151/162	127/147	127/147
SW961	HEX	60.6	141	141	141	113	141	141
SW2174	TET	58.7	101/105	103/105	103/105	101/103	103/109	103/109
SW1473	TET	57.1	171	171/173	171/173	173	171/173	171/173
SW288	TET	55.1	126/134	124/132	124/132	124/132	124/134	124/134
SW66	FAM	54.9	106/110	119/125	119/125	115/119	106/119	106/119
SW1510	HEX	54.3	135	139	139	135	139/144	139/144
SW1856	FAM	54.0	196/198	190/196	190/196	196	196	198

[a]For each microsatellite marker, genotype was determined by size (bp). Litter 1 results from SW288, SW66, and SW1510 and litter 2 results from SWR136, SWR308, SW961, and SW1510 provide the strongest support for the genetic identity of the donor cells and the nuclear transfer piglets

Table 3. Average cell number of day 7 embryos produced in vitro

Embryo type	Average	Range cell number
Nuclear transfer	66	16–125(n = 24)
In vitro fertilized	68	34–124(n = 16)
Activation control	49	13–132(n = 63)
In vivo	-	200–300(refs 23,24)

major difference between the methods described here and those used to produce cloned piglets in two recent reports[12,13] is the use of in vitro matured oocytes from sows rather than in vivo oocytes derived from gilts. Advantages of in vitro matured oocytes include the large number of oocytes that can be obtained from abattoir ovaries and the ability to tightly control the maturity of the oocytes.

Development of porcine NT embryos to blastocyst when cultured for seven days was 7% (72/995). In contrast, cultured IVF embryos and parthenogenetically activated oocytes under the same conditions developed to the blastocyst stage at a rate of 19% (270/1401) and 23% (235/1028), respectively. Table 3 shows that the average cell number in NT blastocysts was comparable to IVF blastocysts but both were higher than parthenogenetic blastocysts. However, the cell number of both NT and IVF blastocysts was approximately one-fourth the estimated cell number for day 7 in vivo embryos (66 cells for NT and IVF in vitro blastocysts versus 200–300 cells for in vivo blastocysts)[23,24].

Pregnancy initiation in the pig requires a critical minimum signal from the embryos to the mother on day 12 of gestation. Polge *et al.*[25] showed that four embryos were minimally required to initiate a pregnancy that would develop to term. If pregnancy initiation depends on total embryo cell number, 16 NT embryos may be needed to produce a pregnancy signal equivalent to four in vivo embryos[26,27]. In addition, if other characteristics of NT embryos such as the inner cell mass:trophectoderm ratio determine embryo viability[28], a given pool of NT embryos probably contains the equivalent of only a few viable embryos. The large number of embryos (115–164; Table 1) that were used to produce the cloned piglets and additional pregnancies described here is consistent with low viability of NT embryos.

Evidence for the inadequacy of in vitro culture systems for porcine embryos is the paucity of reports documenting the birth of piglets from IVF blastocysts[8]. The low cell number of cultured NT, IVF, and parthenogenetic blastocysts as compared to in vivo blastocysts probably reflects these inadequacies. However, six of the seven pregnancies described here were derived from NT embryos cultured for ≥72 h before transfer into recipients despite the low rate of development to blastocyst and low cell number for comparable embryos cultured for seven days. Embryos that produced the cloned piglets recently reported by other groups[12,13] were cultured for approximately 24 and 40 h, respectively.

Low rates of NT embryo development may also reflect inadequate activation. The strategy of Polejaeva et al.[12] using double nuclear transfer, the second round being the transfer of NT pronuclei into enucleated IVF zygotes, was meant to circumvent the need for artificial activation protocols. However, in our experiments, the exposure of porcine cytoplasmic hybrids to higher concentrations of ionomycin (15 μM) for a longer period of time (20 min), compared with typical bovine activation protocols[29,30] was positively correlated with porcine NT development to blastocyst, blastocyst cell number, and pregnancy initiation (unpublished data).

The most immediate application of the donor cell culture and NT procedures that we describe here is in the production of genetically modified pigs for xenotransplantation. The production of CD55 transgenic pigs[31] has allowed the most aggressive form of rejection, HAR, to be overcome[32]. However, xenografts are still rejected by mechanisms that include antibodies, endothelial cell activation, thrombosis, and cellular infiltration. Nuclear transfer can facilitate the production of porcine xenografts in two ways. First, the generation of transgenic pigs will become significantly more efficient, particularly in that transgene integration can be evaluated in vitro preceding NT. The evaluation of transgenes using the existing microinjection technology requires the generation of multiple transgenic lines and expanded breeding programs, a process that can take several years. Second, the cell culture and NT procedures that we have developed should enable gene targeting in the pig. This will allow the inactivation of endogenous genes that are an impediment to long-term xenograft survival and, furthermore, the deletion of endogenous porcine retroviruses that are perceived to be a potential health risk. In summary, the protocol for porcine NT that we describe represents a step forward in the advancement of xenotransplantation.

Experimental protocol

Oocyte collection and maturation. Sow and gilt ovaries were collected at separate local abattoirs and maintained at 30°C during transport to the laboratory. Follicles ranging from 2 to 8 mm were aspirated into 50 ml conical centrifuge tubes (BD Biosciences, Franklin Lakes, NJ) using 18-gauge needles and vacuum set at 100 mm of mercury. Follicular fluid and aspirated oocytes from sows and gilts were pooled separately and rinsed through EmCon filters (Iowa Veterinary Supply Company, Iowa Falls, IA) with HEPES-buffered Tyrodes solution (TL-HEPES) (Biowhittaker, Walkersville, MD). Oocytes surrounded by a compact cumulus mass were selected and placed into North Carolina State University (NCSU) 37 oocyte maturation medium[33] supplemented with 0.1 mg/ml cysteine[34], 10 ng/ml epidermal growth factor[35], 10% porcine follicular fluid[36], 50 μM 2-mercaptoethanol, 0.5 mg/ml cAMP[37], 10 IU/ml each of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG)[38] for ~22 h in humidified air with 5% CO_2 at 38.5°C. Subsequently, the oocytes were moved to fresh NCSU 37 maturation medium that did not contain cAMP, PMSG, or hCG and incubated for an additional 22 h. After ~44 h in maturation medium, oocytes were stripped of their cumulus cells by vortexing in 0.1% hyaluronidase for 1 min.

Donor cells. Day 41 to day 56 porcine fetuses (Yorkshire/Landrace/Newsham sow cross bred to Newsham boar) were collected from pregnant gilts. The intact uterus was excised from the gilt and immediately transported to the laboratory for recovery of fetuses. Fetal gender, weight, crown-rump length, and individual identification were recorded before dissection. Genital ridge cells were obtained by 0.3% protease (from *Streptomyces griseus*) digestion of the genital ridges for 45 min at 37°C. Body cells were obtained from a partial body trypsin–EDTA (Life Technologies, Rockville, MD) digest for 45 min at 37°C. Following digestion, cells were filtered through a 70 μm cell strainer (BD Biosciences), counted and cultured (1.5×10^5 cells per 35 mm culture dish) in high-glucose Dulbecco modified Eagle medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 0.1 mM 2-mercaptoethanol. Typically donor cells were passaged into four-well plates and cultured until confluent. Immediately before nuclear transfer, confluent donor cells in one well were dissociated by incubation with 0.1% protease for ~10 min, washed once with TL-HEPES supplemented with 10% FBS, collected by centrifugation for 10 min at 250 *g*, and resuspended in ~0.5 ml Dulbecco's phosphate-buffered saline (DPBS; Life Technologies).

Nuclear transfer. On removal of cumulus cells, oocytes were placed in NCSU 23 embryo culture medium[39] that contained 1 μg/ml Hoechst 33342 and 7.5 μg/ml cytochalasin B for ~30 min. Micromanipulation of donor cells and oocytes was performed in drops of TL-HEPES on 100 mm dishes (BD Biosciences) covered with light mineral oil. Glass capillary microtools were produced using a pipette puller (Sutter Instruments, Novato, CA) and microforge (Narishige International, East Meadow, NY). Metaphase II oocytes were enucleated by removal of the polar body and the associated metaphase plate. Absence of the metaphase plate was visually verified by ultraviolet fluorescence, keeping exposure to a minimum. A single donor cell was placed in the perivitelline space of the oocyte so as to contact the oocyte membrane. An electrical pulse of 99 volts for 45 μs from an ElectroCell Manipulator 200 (Genetronics, San Diego, CA) was used to fuse the membranes of the donor cell and oocyte, forming a cytoplasmic hybrid. The fusion chamber consisted of wire electrodes 500 μm apart and the fusion medium was SOR2 (0.25 M sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% BSA (Fraction V; Sigma, St. Louis, MO); pH 7.2 and osmolarity 250 mOsm). Following the fusion pulse, cytoplasmic hybrids were incubated in CR2 embryo culture medium[39] for approximately 4 h before activation.

Activation and embryo culture. Cytoplasmic hybrids were activated by incubation in 15 μM calcium ionomycin (Calbiochem, San Diego, CA) for 20 min followed by incubation with 1.9 mM 6-dimethylaminopurine (DMAP) in CR2 medium for 3–4 h. After DMAP incubation, cytoplasmic hybrids were washed through two 35 mm dishes containing TL-HEPES, cultured in CR2 medium containing 3 mg/ml BSA for 48 h, placed in NCSU 23 medium containing 0.4% BSA for 24 h, and further cultured in NCSU 23 containing 10% FBS. Embryos that developed to blastocyst stage by day 7 in vitro were fixed in 4% paraformaldehyde, stained with Hoechst 33342, and placed under coverslips on glass slides. Fixed embryos were visualized with ultraviolet fluorescence and cells were counted.

In vitro fertilization. Fifty matured oocytes, stripped of their cumulus and in a volume of 3 μl, were placed into 92 μl drops of fertilization medium (TLP-PVA)[40,41]. Each drop containing oocytes was inseminated with 5 μl of fertilization medium containing 2,000 sperm. Fresh boar semen was purchased from Genes Diffusion (Stoughton, WI). Several different boars were used during the course of these experiments. Following 10 min of co-incubation with sperm, the oocytes were moved to a fresh drop of fertilization medium and incubated for an additional 5 h. Oocytes were washed through unused fertilization drops to remove sperm and cultured in NCSU 23 with 0.4% BSA until embryos were transferred into recipients 0–3 days after fertilization. Embryos cultured to evaluate development rates were placed in NCSU 23 medium with 10% FBS from day 5 to day 7.

Embryo transfer into recipients. Embryos at various stages of development were surgically transferred into uteri of asynchronous recipients[42]. Recipient females (parity 0 or 1) were selected that exhibited first standing estrus 24 h preceding oocyte activation. For surgical embryo transfer, anesthesia was induced with a combination of the following: ketamine, 2 mg/kg; tiletamine, 0.25 mg/kg; zolazepam, 0.25 mg/kg; xylazine, 1 mg/kg; and atropine, 0.03 mg/kg (Iowa Veterinary Supply). Anesthesia was maintained with 3% halothane. While in dorsal recumbency the recipients were aseptically prepared for surgery and a caudal ventral incision was made to expose and examine the reproductive tract. Embryos that were cultured ≤48 h (one- to two-cell stage) were placed in the ampullar region of the oviduct by feeding a 5.5-inch Tomcat catheter (Sherwood Medical, St. Louis, MO) through the ovarian fimbria. Embryos cultured 48 h or more (at or greater than four-cell stage) were placed in the tip of the uterine horn. Typically, 100–300 NT embryos were placed in the oviduct or uterine tip depending on embryonic stage, and 100 IVF embryos were placed in the oviduct. All protocols conformed to University of Wisconsin animal health care guidelines. Pregnancies were detected using an Aloka 500 ultrasound scanner (Aloka Co. Ltd., Wallingford, CT) with an attached 3.5 MHz transabdominal probe. Monitoring for pregnancy initiation began 23 days after fusion/fertilization and was repeated as necessary through day 40. Pregnant recipients were examined by ultrasound weekly.

Parentage analysis. For each PCR reaction, 60 ng of porcine genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM forward primer, 1 μM reverse primer, and 0.6 units of AmpliTaq DNA polymerase (PE Biosystems, Foster City, CA) were used in a total volume of 15 μl. Primers were one of 11 microsatellites labeled with either of the fluorescent dyes FAM, TET, or HEX (ref. 43). Thermal cycling was performed in a 96-well plate using an MJ Research PTC-225 Tetrad thermal cycler (MJ Research, Waltham, MA). Following initial denaturation of 3 min at 95°C, DNA was amplified in 35 cycles of polymerase chain reaction (1 min at 95°C, 30 s at an appropriate annealing temperature and 1 min at 72°C) and completed with a final elongation step of 4 min at 72°C. After cycling, an aliquot of each reaction was combined with an internal size standard (Genescan 350, PE Biosystems) and loaded onto an ABI 377 (PE Biosystems) automated fluorescent DNA sequencer for electrophoretic separation. Samples were tracked and analyzed using Genescan version 3.1 and Genotyper version 3.6 NT (PE Biosystems).

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Sheep cloned by nuclear transfer from a cultured cell line

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NUCLEAR transfer has been used in mammals as both a valuable tool in embryological studies[1] and as a method for the multiplication of 'elite' embryos[2–4]. Offspring have only been reported when early embryos, or embryo-derived cells during primary culture, were used as nuclear donors[5,6]. Here we provide the first report, to our knowledge, of live mammalian offspring following nuclear transfer from an established cell line. Lambs were born after cells derived from sheep embryos, which had been cultured for 6 to 13 passages, were induced to quiesce by serum starvation before transfer of their nuclei into enucleated oocytes. Induction of quiescence in the donor cells may modify the donor chromatin structure to help nuclear reprogramming and allow development. This approach will provide the same powerful opportunities for analysis and modification of gene function in livestock species that are available in the mouse through the use of embryonic stem cells[7].

The cells used in these experiments were isolated by microdissection and explantation of the embryonic disc (ED) of day 9 *in vivo* produced 'Welsh mountain' sheep embryos. The line was established from early passage colonies with a morphology like that of embryonic stem (ES) cells. By the second and third passages, the cells had assumed a more epithelial, flattened morphology (Fig. 1*a*) which was maintained on further culture (to at least passage 25). At passage 6, unlike murine ES cells they expressed cytokeratin, and nuclear lamin A/C which are markers associated with differentiation[8]. This embryo-derived epithelial cell line has been designated TNT4 (for totipotent for nuclear transfer).

The development of embryos reconstructed by nuclear transfer is dependent upon interactions between the donor nucleus and the recipient cytoplasm. We have previously reported the effects of the cytoplasmic kinase activity, maturation/mitosis/meiosis promoting factor (MPF), on the incidence of chromosomal damage and aneuploidy in reconstructed embryos and established two means of preventing such damage[9]. First, the effects of the donor cell-cycle stage can be overcome by transferring nuclei after the disappearance of MPF activity by prior activation of the recipient enucleated MII oocyte[9,10]. Using this approach we obtained the birth of lambs by nuclear transfer during establishment of the cell line (up to and including passage 3). On subsequent culture (passages 6 and 11) no development to term was obtained (see Table 1). From these numbers we cannot conclude that development to term will not be obtained using this method. The lack of development of some control embryos is thought to relate to an infection in the oviduct of the temporary recipient ewe from which 6 were recovered.

An alternative means of avoiding damage due to the activity of MPF is to transfer diploid nuclei into metaphase II oocytes that have a high level of MPF activity[9]. The availability of TNT4 cells allows this approach to be used. In this study a synchronous population of diploid donor nuclei was produced by inducing the cells to exit the growth cycle and arrest in G0 in a state of quiescence. In the presence of a high level of MPF activity the transferred nucleus undergoes nuclear membrane breakdown and chromosome condensation. It has been argued[11] that the developmental potential of reconstructed embryos depends upon the "reprogramming of gene expression" by the action of cytoplasmic factors and that this might be enhanced by the prolongation of this period of exposure. To assess these effects donor cells were fused to oocytes either (1) 4–8 h before activation 'post-activated' or (2) at the time of activation 'fusion and activation' or (3) to pre-activated oocytes 'preactivated'.

During these studies *in vivo* ovulated metaphase arrested (MII) oocytes were flushed from the oviduct of 'Scottish blackface' ewes. The methodology used was as previously described[10] with the following exceptions; oocytes were recovered 28–33 h after injection of gonadotropin-releasing hormone (GnRH), calcium/magnesium-free PBS containing 1.0% FCS was used for all flushing, and recovered oocytes were transferred to calcium-free M2 medium[12] containing 10% FCS and were maintained at 37 °C in 5% CO_2 in air until use. As soon as possible after recovery oocytes were enucleated and embryos reconstructed. At 50–54 h

TABLE 1 Development using unsynchronized TNT4 cells

Donor cell type	Number of morula and blastocysts/total embryos (%)	Number of lambs/ embryos transferred
	October 1993–February 1994	
16 cell	6/11 (27.3)	2/6
ED cell	1/15 (6.7)	0/1
ED P1	4/19 (21.0)	1/4
ED P2	1/11 (9.1)	1/1 *
ED P3	2/36 (5.5)	2/2
	October–December 1994	
16 cell	14/28 (50.0)	0/14
TNT4 P6	9/98 (9.2)	0*/9
TNT4 P11	10/92 (10.9)	0/10

Development of ovine embryos reconstructed by nuclear transfer of unsynchronized cells during isolation and after establishment of the TNT4 line to enucleated preactivated ovine oocytes. P, Passage number; ED, embryonic disc. For embryo reconstruction, donor oocytes were placed into calcium-free M2 containing 10% FCS, 7.5 μg ml^{-1} Cytochalasin B (Sigma) and 5.0 μg ml^{-1} Hoechst 33342 (Sigma) at 37 °C for 20 min to aspirate. A small amount of cytoplasm enclosed in plasma membrane was removed from directly beneath the 1st polar body using a glass pipette (~20 μm tip external diameter). Enucleation was confirmed by exposing this karyoplast to ultraviolet light and checking for the presence of a metaphase plate. At 34–36 h after GnRH injection enucleated oocytes were activated. Following further culture for 4–6 h in TC199, 10% FCS a single cell was fused. All activations and fusions were accomplished as previously described[10,17] in 0.3 M mannitol, 0.1 mM $MgSO_4$, 0.0005 mM $CaCl_2$[17]. For activation a single DC pulse of 1.25 kV cm^{-1} for 80 μs and for fusion an AC pulse of 3 V for 5 s followed by 3 d.c. pulses of 1.25 kV cm^{-1} for 80 μs were applied. All oocyte/cell couplets were cultured in TC199, 10% FCS 7.5 μg ml^{-1} Cytochalasin B (SIGMA) for 1 h following application of the fusion pulse and then in the same medium without Cytochalasin until transferred to temporary recipient ewes. Reconstructed embryos were cultured in the ligated oviduct of a recipient 'blackface' ewe until day 7 after reconstruction. All morula and blastocyst stage embryos were transferred to synchronized recipient blackface ewes for development to term.

* A single pregnancy was established but subsequently lost at about 70–80 days.

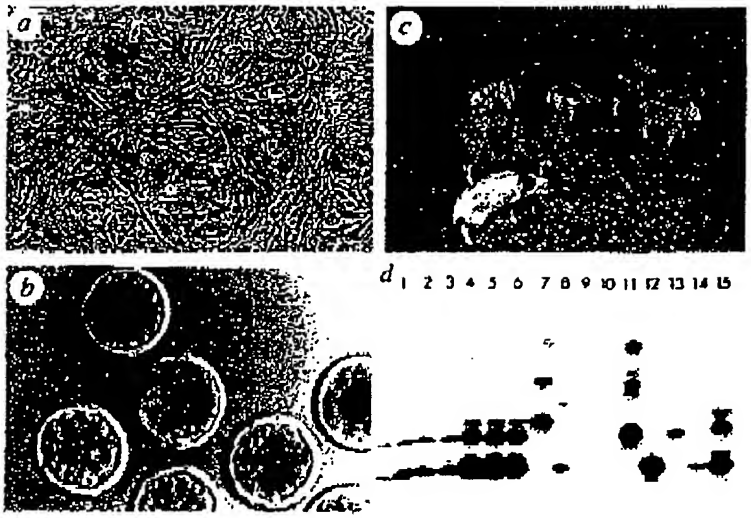

FIG. 1 Production and characterization of the TNT4 cell line and the offspring produced by nuclear transfer from TNT4 cells. *a*, Morphology of the TNT4 cell line at passage 6. *b*, Group of embryos including a single blastocyst on day 7 after reconstruction. *c*, Group of three Welsh mountain lambs produced by nuclear transfer with surrogate Scottish blackface ewes. *d*, Autoradiogram showing the alleles generated following amplification of the microsatellite FCB266 (ref. 18). Lanes 1–6 are from, respectively, TNT4 cells and the five lambs generated by nuclear transfer. Both lambs and cells display an identical pattern, revealing 2 alleles (arrowed) at 114 and 125 bp. Lanes 7–15, nine randomly chosen Welsh mountain sheep, none of whom show an identical pattern to the nuclear transfer group. Lambs and TNT4 cells were also identical at six further microsatellite loci: MAF33, MAF48, MAF65, MAF209, OarFCB11, OarFCB128, OarRCB304 (data not shown). The nine unrelated random control animals showed extensive variation at all of these loci.

METHODS. Groups of 4–6 microdissected embryonic discs were cultured on feeder layers of mitotically inactivated primary murine fibroblasts in Dulbeccos Modified Eagles medium (GIBCO) containing 10% fetal calf serum, 10% newborn serum and supplemented with recombinant human leukaemia inhibition factor (LIF). After 5–7 days of culture, expanding discs were treated with trypsin and passaged onto fresh feeders yielding 4 similar lines. At passage 12 of the 2*n* chromosome complement of 54 was observed in 31 of 50 spreads, the remaining aneuploid spreads are thought to be artefacts of preparation. For microsatellite analysis genomic DNA was extracted from whole blood, tissue culture cells or fetal tissues using a puregene DNA isolation kit (Gentra Systems Inc., Minneapolis, USA). The PCR analysis of microsatellites was carried out using an end-labelled primer ([γ-^{32}P]ATP). All other aspects of labelling and thermal cycling conditions were as described elsewhere[17].

after GnRH injection, reconstructed embryos were embedded in agar and transferred to the ligated oviduct of dioestrus ewes. After 6 days the embryos were retrieved and development assessed microscopically (see Fig. 1*b*).

The development of embryos reconstructed using quiescent TNT4 cells and 3 different cytoplast recipients is summarized in Table 2. No significant difference was observed in the frequency of development with high and low passage number donor cells or with cytoplast recipient type used (results were analysed by the marginal model in ref. 13). All embryos that had developed to the morula/blastocyst stage were transferred as soon as possible to the uterine horn of synchronized final recipient ewes for development to term. Recipient ewes were monitored for pregnancy by ultrasonography. Ewes that were positive at day 35 were classified as pregnant (Table 3). A total of eight fetuses were detected in seven recipient ewes including a single twin pregnancy. A total of five phenotypically female Welsh mountain lambs were born from the Scottish blackface recipient ewes (Fig. 1*c*). Two of these lambs died within minutes of birth and a third at 10 days; the remaining two lambs are apparently normal and healthy (8–9 months old). Of the remaining 3 fetuses, one was lost at about 80 days of gestation, and a second was lost at 144 days of gestation. The third fetus was thought to be a twin pregnancy and was either misdiagnosed or lost at an unknown time. Microsatellite analysis of the cell line, fetuses and lambs showed that all of the female lambs were derived from a single cell population (Fig. 1*d*).

TABLE 2 Development to morula and blastocyst stage of ovine embryos reconstructed using quiescent TNT4 cells and 3 different cytoplast recipients (January–March 1995)

Experiment number	Cytoplast type: TNT passage number	Number of morulae and blastocysts/total number of embryos recovered (%): Post-activated	Activation and fusion	Preactivated
1	6	4/28	6/32*	–
2	7	1/10	1/26*	–
3	13	0/2	–	2/14
4	13	0/14	0/11	–
5	11	1/9	–	0/9
6	11	1/2	9/29***	–
7	12	–		6/45*
8	13	3/13*		–
Total		10/78 (12.8%)	16/98 (16.3%)	8/68 (11.7%)

Development to the morula and blastocyst stage of ovine embryos recovered on day 7 after reconstruction by nuclear transfer of quiescent TNT4 cells at different passages into 3 cytoplast recipients. To induce quiescence, TNT4 cells were plated into feeder layers in 29-cm^2 flasks (GIBCO) and cultured for 2 days, the semiconfluent exponentially growing cultures were then washed three times in medium containing 0.5% FCS and cultured in this low-serum medium for 5 days. Embryos were reconstructed using preactivated cytoplasts as previously described (Table 1) and by two other protocols. (1) post-activation, as soon as possible after enucleation a single cell was fused to the cytoplast in 0.3 M mannitol without calcium and magnesium, to prevent activation. Couplets were washed and cultured in calcium-free M2, 10% FCS at 37 °C, 5% CO_2 for 4–8 h. Thirty minutes before activation the couplets were transferred to M2 medium, 10% FCS containing 5 μM Nocodazole (SIGMA). Following activation the reconstructed zygotes were incubated in medium TC199, 10% FCS, 5.0 μM Nocodazole for a further 3 h. (2) Preactivation, at 34–36 h after GnRH injection a single cell was fused to an enucleated oocyte. The same pulse also induced activation of the recipient cytoplast. All activations and fusions were accomplished as described in Table 1 unless otherwise stated.

* Denotes number of pregnancies following transfer of morula and blastocyst stage embryos to synchronized final recipient ewes.

TABLE 3 Induction of pregnancy and further development following transfer of morula and blastocyst stage embryos reconstructed from quiescent TNT4 cells

Cytoplast type	Post-activated	Activation and fusion	Preactivated
Total number of morula and blastocyst stage embryos transferred	10	16	8
Total number of ewes	6	9	4
Number of pregnant ewes (%)	1 (16.7)	5 (55.5)	1 (25.0)
Number of fetuses/total embryos transferred (%)	2/10 (20.0)	5/16 (31.25)	1/8 (12.5)
Number of live births	1	3	1
Passage number of cells resulting in offspring	1 × P11	1 × P6, 2 × P11	1 × P13

Induction of pregnancy following transfer of all morula/blastocyst stage reconstructed embryos to the uterine horn of synchronized final recipient blackface ewes. The table shows the total number of embryos from each group transferred, the frequency of pregnancy in terms of ewes and embryos (in the majority of cases 2 embryos were transferred to each ewe and a single twin pregnancy was established (using the 'post-activated' cytoplast)) and the number of live lambs obtained.

Because of the seasonality of sheep a direct comparison of all of these methods of embryo reconstruction has not yet been made. The success of the later studies may be due to a number of factors. First, quiescent nuclei are diploid and therefore the cell-cycle stages of the karyoplast and cytoplast in both the 'post-activation' and 'fusion and activation' methods of reconstruction are coordinated. The preactivated cytoplast will accept donor nuclei from G0, G1, S and G2 cell-cycle phases. Second, the G0 phase of the cell cycle has been implicated in the differentiation process and the chromatin of quiescent nuclei has been reported to undergo modification[14]. As a result the chromatin of quiescent donor nuclei may be more readily modified by oocyte cytoplasm. The TNT4 cells resemble several cell lines derived previously in sheep[15] and also pigs[16]. It remains to be determined whether comparable development is obtained with other such lines or other cell types. At the present time we are unable to differentiate the mechanisms involved and report that the combination of nuclear transfer and cell type described here support development to term of cloned ovine embryos from cells that had been in culture through up to 13 passages. As cell-cycle duration was about 24 h, this period of culture before nuclear transfer would be sufficient to allow genetic modification and selection if procedures comparable to those used in murine ES cells can be established.

The production of cloned offspring in farm animal species could provide enormous benefits in research, agriculture and biotechnology. The modification by gene targeting and selection of cell populations before embryo reconstruction coupled to the clonal origin of the whole animal provides a method for the dissemination of rapid genetic improvement and/or modification into the population □

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Midbrain development induced by FGF8 in the chick embryo

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Vertebrate midbrain development depends on an organizing centre located at the isthmus, a constriction in the embryonic mid/hindbrain region[1–3,28]. Isthmic tissue grafts transform chick caudal forebrain into an ectopic midbrain that is the mirror image of the normal midbrain[4]. Here we report that FGF8 protein has the same midbrain-inducing and polarizing effect as isthmic tissue. Moreover, FGF8 induces ectopic expression in the forebrain of genes normally expressed in the isthmus, suggesting that the ectopic midbrain forms under the influence of signals from a new 'isthmus-like' organizing centre induced in the forebrain. Because *Fgf8* itself is expressed in the isthmus, our results identify FGF8 as an important signalling molecule in normal midbrain development.

Fgf8 is expressed in the isthmus of the developing mouse brain[5–8]. Because FGF8 has inducing activity in another developmental system (the limb[9]), we sought to determine whether FGF8 provides the midbrain-inducing activity of an isthmus graft in the chick. We first confirmed that *Fgf8* is expressed in the chick isthmus (Fig. 1*a*). Next, we determined the effects of implanting a bead soaked in recombinant FGF8 (FGF8-bead) into the caudal diencephalon (prosomere 2, p2, as defined in ref. 10; Fig. 1*b*) of chick embryos at stages 9–12 (ref. 11). An early effect of isthmus grafts is induction in the host neuroepithelium of *Engrailed*-2 (*En2*) expression[1,4], an early marker of mes/rhombencephalic development[12–14]. When an FGF8-bead was implanted, ectopic *En2* RNA was detected caudal to the zona limitans intrathalamica (ZL), a transverse boundary separating dorsal and ventral thalamus anlagen (p2/p3 boundary[10]), in all embryos assayed 22–26 h later ($n = 10$; Fig. 1*c*). Control beads soaked in phosphate-buffered saline (PBS-beads) did not induce *En2* expression ($n = 11$; not shown).

In experimental embryos surviving to E5–E16 (stages 25–42) the diencephalon caudal to the ZL (p1 and p2; ref. 10) was transformed from its normal fate of rostral pretectum and dorsal thalamus to ectopic midbrain ($n = 17/18$; Fig. 2). Control embryos implanted with PBS-beads that survived to E4–E10

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Efficient Human Sperm Pronucleus Formation and Replication in *Xenopus* Egg Extracts[1]

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ABSTRACT

We have achieved efficient in vitro reactivation and replication of human sperm nuclei in frog egg extracts by constructing a 4-step protocol that mimics the events of fertilization and pronucleus formation in mammalian eggs. With use of this protocol, 78–97% of human sperm nuclei from fertile donors synchronously swelled and completed full genome replication in about 2 h. We document the changes in nuclear structure that accompany efficient DNA synthesis and discuss future research and potential clinical implications of this new system.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) involves injection of an entire sperm cell directly into the cytoplasm of an unfertilized egg [1]. The worldwide use of ICSI and the resulting births of many healthy babies demonstrate that this procedure works in humans and is an effective treatment of male factor infertility, despite some concerns [2]. It is not known, however, why ICSI results in reactivation of the human sperm nucleus since this procedure bypasses the normal pathway of sperm-egg fusion and contact of the sperm nucleus-and-cytoskeleton with the subcortical cytoplasm of the egg. These steps appear to be critical for bovine fertilization [3], and ICSI does not work in cows and other farm animals [4]. In order to understand the mechanism of ICSI in humans, it is necessary to develop experimental systems that permit detailed analysis of the events of human sperm pronucleus formation.

Given these considerations, we have built an in vitro system for efficient human pronucleus formation and replication. The process of human sperm pronucleus formation has previously been analyzed in intact hamster eggs [5] and in several in vitro systems using *Xenopus* egg extracts [6–9]. The system we describe here also utilizes *Xenopus* egg extracts, but it is more efficient and synchronous than previous systems. The key difference is that our system uses two different *Xenopus* egg extracts: one prepared from unactivated *Xenopus* eggs arrested in meiotic metaphase II (MII-Extract), and a second prepared from activated eggs at the peak of their DNA synthetic capacity for human sperm pronuclei (Interphase-Extract). This two-cytoplasm approach mimics the events of fertilization. During normal fertilization, the sperm nucleus with its surrounding cytoskeleton contacts the cytoplasm of the egg while the egg is still arrested in meiotic metaphase II. The egg then reenters the cell cycle, and its cytoplasm advances into the interphase state. Earlier studies from our laboratory have demonstrated that the two-cytoplasm approach results in efficient plasmid DNA synthesis in intact *Xenopus* eggs [10] and egg extracts [11], as well as efficient reactivation and replication of *Xenopus* red blood cell nuclei, which, like sperm nuclei, are quiescent and highly condensed [12, 13]. The two-cytoplasm approach is also the basis for several recent successes in mammalian cloning [14–16], in accord with our predictions [13].

MATERIALS AND METHODS

Preparation of Xenopus Eggs

Female frogs, obtained from Nasco (Fort Atkinson, WI), were cared for and ovulated as described previously, and the resulting eggs were hardened and used to prepare both high-speed MII-Extract (previously called CSF-Extract) and low-speed Interphase-Extract (previously called Activated-Extract) as previously described [13, 17]. The eggs used for preparation of Interphase-Extract were activated by calcium ionophore treatment and incubated at 20°C for a total of 22 min, rather than 28 min, before being centrifuged (see *Results*). The kinetics of cell cycle progression at 20°C has been previously established [10, 13].

Preparation of Human Sperm

Frozen samples of sperm from fertile donors and subfertile patients were provided by Boston Fertility Laboratories, Inc. (Brookline, MA). Samples of sperm were obtained from fertile sperm donors and from subfertile infertility patients. The sperm donors were contributors to the Boston Fertility Laboratory sperm bank whose sperm had been used to achieve ongoing pregnancies through in vitro fertilization or intrauterine insemination. These samples had prefreezing sperm counts between 60 and 70 million/ml, a sperm morphology range of 6–14% normal forms, and at least 50% motility using strict criteria developed at Boston Fertility Laboratory. The subfertile males were from couples seeking infertility treatment due to failure to achieve pregnancy after unprotected intercourse for a least one year. The primary diagnosis in each case was male factor infertility, and all samples showed less than 10% normal morphology readings. All donors and patients gave informed consent for use of their sperm according to the guidelines approved by the Committee on Clinical Investigations at the Beth Israel Deaconess Hospital, Boston, MA. Sperm morphology was assessed using the strict criteria implemented by Boston Fertility Laboratories, Inc.

All samples were first allowed to liquefy and were then mixed with an equal volume of TEST Yolk buffer (Irvine Scientific, Santa Ana, CA) before being frozen by exposing aliquots to liquid nitrogen vapor for 2 h followed by immersion in liquid nitrogen. As needed, sperm samples were

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thawed on ice and washed twice by suspension and centrifugation in cold nuclear isolation buffer, NIB (250 mM sucrose, 25 mM NaCl, 10 mM PIPES, 1.5 mM $MgCl_2$, 0.5 mM spermidine, 0.15 mM spermine, pH 7.0) for 15 min at 1200 × *g* at 4°C. The resulting pellet was resuspended in NIB, and aliquots containing 3×10^7 cells in 200 μl were then refrozen in liquid N_2 in siliconized tubes.

In Vitro Reactivation of Human Sperm Nuclei

Chemical pretreatment. Individual aliquots of human sperm were thawed on ice and were permeabilized by incubation in 100 μg/ml of lysolecithin in NIB for 5 min at 25°C in a final volume of 1 ml. Lysolecithin treatment was then stopped by addition of BSA and soybean trypsin inhibitor to final concentrations of 0.4% (w:v) and 30 μg/ml, respectively, and samples were centrifuged at 1200 × *g* for 20 min at 2°C. The pellet was washed once in 1 ml of 0.4% BSA in NIB. After removal of the supernatant, the pellet was incubated for 20 min at 25°C in 400 μl NIB containing 5 mM dithiothreitol (DTT). DTT reduction was stopped by addition of *N*-ethylmaleimide to a 1 mM concentration and incubated for 10 min at 25°C. Samples were centrifuged at 1200 × *g* for 20 min at 2°C, and each pellet was resuspended in NIB to a final concentration of 2×10^4 sperm/μl.

Further pretreatment in MII-Extract. Frozen aliquots of MII-Extract were thawed on ice and were supplemented with 1/10th volume of a 10-strength ATP-regenerating mix (single-strength: 0.4 mM creatine phosphate, 0.4 μg/ml creatine phosphokinase, 0.1 mM $CaCl_2$ in sterile water). Pretreated sperm were then diluted 1:5 into the extract to a final concentration of 4000 sperm/μl. The reaction was incubated at 25°C for 10–18 min, depending on the MII-Extract, until the sperm had undergone maximum swelling as observed by fluorescent staining with Hoechst 33342. The sample was then placed on ice for 60 min.

Nuclear activation and replication in Interphase-Extract. The MII-Extract was triggered to enter interphase by addition of 1.2 mM $CaCl_2$ and incubation at 25°C for 10 min; it was then diluted into 9 volumes of Interphase-Extract supplemented with 0.4 mM creatine phosphate and 0.4 μg/ml creatine phosphokinase. The Interphase-Extract was incubated at 25°C for 180 min and throughout this time was sampled at regular intervals by withdrawing 5-μl aliquots. The kinetics of DNA synthesis were determined by incorporation of [α-^{32}P]dCTP followed by gel electrophoresis of the labeled product; DNA replication on a per nucleus level was determined by incorporation of biotinylated dUTP followed by Texas Red/streptavidin staining; quantitation of the extent of DNA synthesis was determined by incorporation of bromo-dUTP (Br-dUTP) followed by CsCl density gradient centrifugation, as previously described [10, 13].

By using Gaussian curve analysis of the peaks resolved in the CsCl density gradient, we determined the percentages of replicated and unreplicated molecules present at the beginning and end of S-phase and used these values to deduce the efficiency of replication [10]. The formula used to quantitate the process of DNA replication was:

$$\frac{\frac{HL}{2}}{\frac{HL}{2} + LL + \frac{4LLH}{3}}$$

HL = once-replicated heavy-light DNA; LL = unreplicated light-light DNA; LLH = partially replicated DNA.

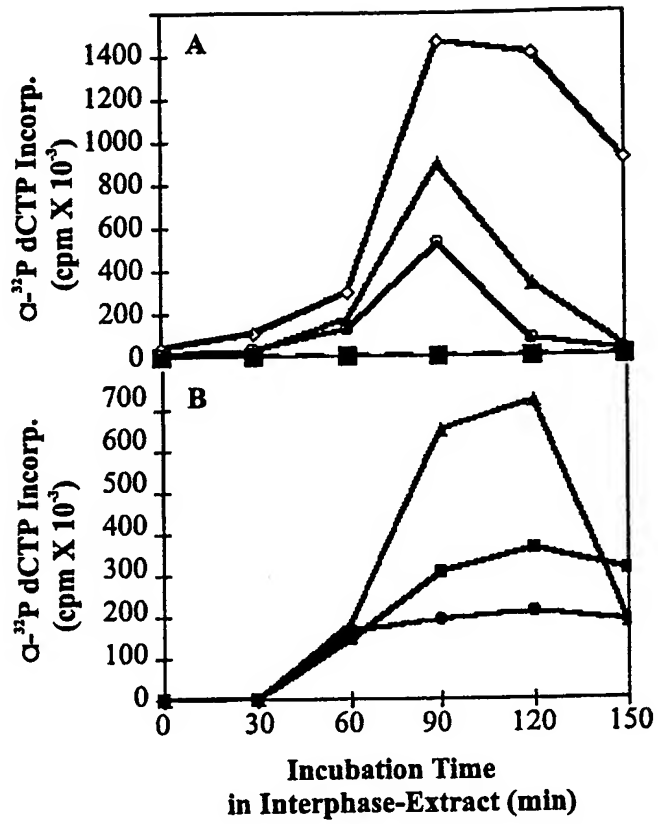

FIG. 1. Preincubation time in MII-Extract was critical for efficient replication. Human sperm were preincubated for increasing times in an MII-Extract and were then assayed for replication upon dilution into Interphase-Extract. The results from 2 representative independently performed experiments are shown in **A** and **B**. In each case, DNA replication was assayed by measuring [α-^{32}P]dCTP incorporation into genomic DNA. **A**) Preincubation times in MII-Extract: 0 min (squares), 15 min (diamonds), 20 min (triangles), and 35 min (circles); **B**) 6 min (circles), 12 min (triangles), 15 min (squares). In addition, DNA replication was not observed in 5 additional experiments in which sperm were not preincubated in MII-Extracts.

Fluorescent Imaging and Deconvolution

Fluorescent images of nuclei stained with Hoechst 33342 were acquired and analyzed using the CELLscan deconvolution system as described previously [18]. In brief, this system collects a series of optical sections at focal planes 0.25 μm apart using a 100×/1.4 N.A. Olympus (Tokyo, Japan) objective connected to a piezoelectric Z-axis focusing device and a computer-controlled excitation light shutter. The set of images is then deconvolved by application of the Exhaustive Photon Reassignment algorithm built into the CELLscan software. This algorithm vectorially reassigns the light haze contributed by fluorescent structures located above and below the plane of optimal focus to its proper places of origin after accurate characterization of the blurring function of the optical system. Measurements of distances in the XY-axis were performed after calibration of the CELLscan software using a CELLscan system stage micrometer as an external metric standard.

RESULTS

Investigations Leading to an Optimized In Vitro System

We set out to develop a 4-step protocol for in vitro reactivation and replication of human sperm that recapitulates the process of pronucleus development in fertilized human

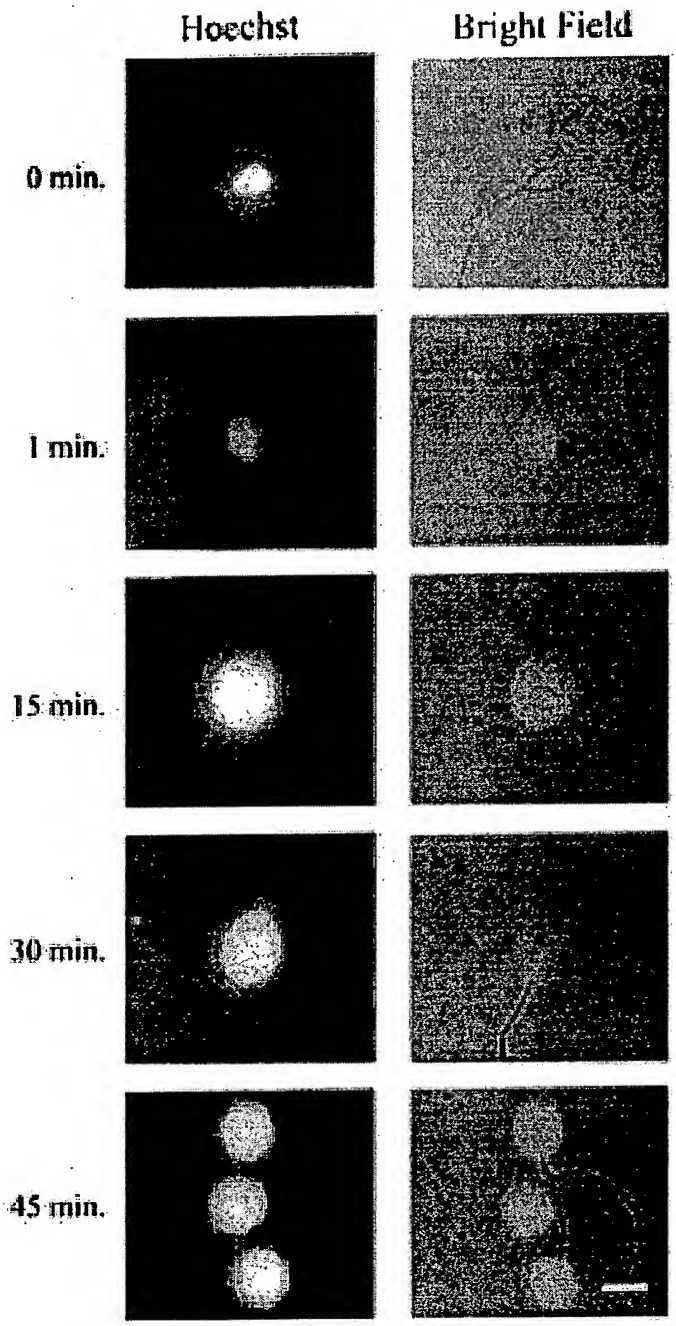

FIG. 2. Morphological changes of human sperm in MII-Extract. Chemically pretreated sperm nuclei were incubated in MII-Extract for the times indicated and were then fixed and stained with Hoechst 33342 for observation with brightfield and fluorescent microscopy. Nuclear envelope breakdown was followed by chromatin decondensation, chromatin recondensation, nuclear ruffling, and eventual dispersion into fibers (not shown; bar = 5 μm).

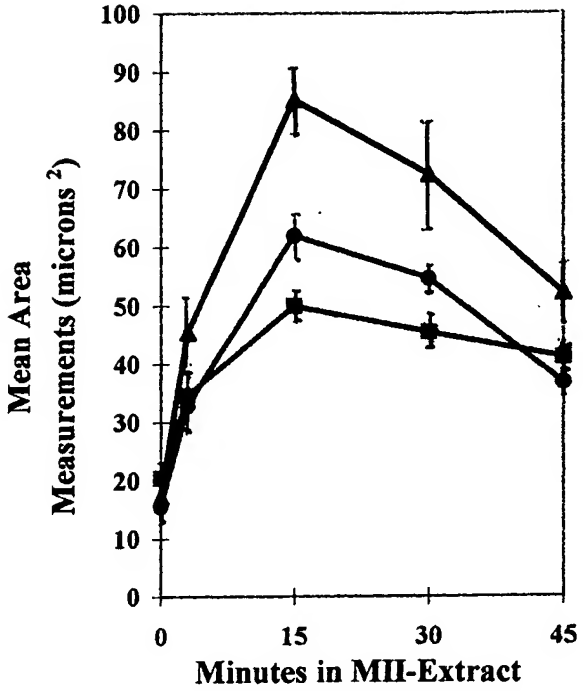

FIG. 3. Area measurements of human sperm incubated in the MII-Extract. Aliquots of chemically pretreated sperm were incubated in MII-Extract, fixed, and then stained with Hoechst 33342. Images were photographed using a charged coupled device camera driven by IPLab Spectrum (Scanalytics, Billerica, MA) software and were analyzed with NIH Image (Bethesda, MD) software. Each data point represents the mean area measurement of approximately 20 sperm nuclei. The results were obtained from 3 independently performed experiments. Error bars indicate the 95% confidence interval for each mean (i.e., mean ± 3 SD).

eggs. In this protocol, intact sperm were first treated with lysolecithin to permeabilize the plasma membrane and then with DTT to disrupt the disulfide bonds between the sperm protamines. Chemically pretreated sperm were then incubated in MII-Extract and next diluted into Interphase-Extract, where pronucleus formation and DNA replication take place. Figure 1 demonstrates that efficient replication in pretreated human sperm nuclei in the Interphase-Extract was critically dependent on a limited period of preincubation in MII-Extract. In 5 separate experiments, sperm samples not preincubated in MII-Extracts failed to replicate DNA upon dilution into Interphase-Extract (data not shown). Cytological examination revealed that human sperm nuclei were still very compact following lysolecithin permeabilization and disulfide reduction, but they swelled very rapidly when they were incubated in MII-Extract (Fig. 2). The point of maximum swelling was reached after 10–18 min (average: 12 min, from 25 separate experiments) depending on the particular batch of MII-Extract. Thereafter, nuclear size decreased slightly and the contour of each nucleus appeared more ragged (Fig. 2). Nuclei eventually formed dispersed chromosomal fibers (data not shown).

Nuclei preincubated in MII-Extract to the point when they first achieved maximum swelling went on to synthesize DNA rapidly once diluted into Interphase-Extract (compare Figs. 1 and 3). In contrast, sperm nuclei not exposed to MII-Extract did not synthesize DNA in Interphase-Extract. Nuclei preincubated in MII-Extract for more than the optimal length of time exhibited lower overall replication and stopped DNA synthesis sooner (compare 15-min and 20-min samples in Fig. 1). Thus, measurement of nuclear area in MII-Extract provided a convenient means of optimizing subsequent replication in Interphase-Extract.

Because activated *Xenopus* eggs progress through the cell cycle very quickly, small differences in the time or temperature at which activated eggs are incubated prior to preparation of Interphase-Extract have major effects on the intrinsic capacity of the extract to support DNA synthesis. For instance, extracts prepared from eggs incubated for 28 min at 20°C after activation with calcium ionophore A23187 exhibit the highest DNA synthetic capacity in *Xenopus* erythrocyte nuclei [13]. In order to establish which Interphase-Extract exhibited the highest capacity for DNA

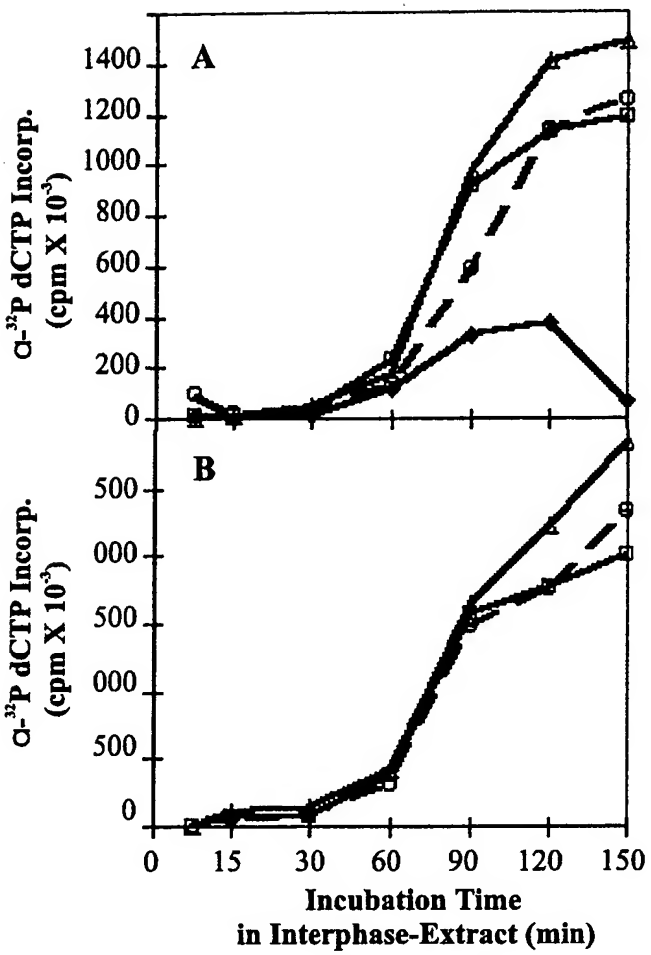

FIG. 4. Preparation of optimal Interphase-Extract. A and B) Results of 2 independently performed experiments using 2 independently prepared sets of Interphase-Extracts. In each case, fully pretreated sperm were diluted into different batches of Interphase-Extract prepared from eggs that had entered the cell cycle for increasing lengths of time at 20°C. A) 12 min (diamonds), 17 min (squares), 22 min (triangles), and 26 min (circles); B) 17 min (squares), 22 min (triangles), and 27 min (circles). DNA replication was assayed by measuring [α-^{32}P]dCTP incorporation. The results of both experiments demonstrated that the extract prepared from eggs activated for 22 min exhibited the highest DNA synthetic capacity when measured over the entire time course of the reaction.

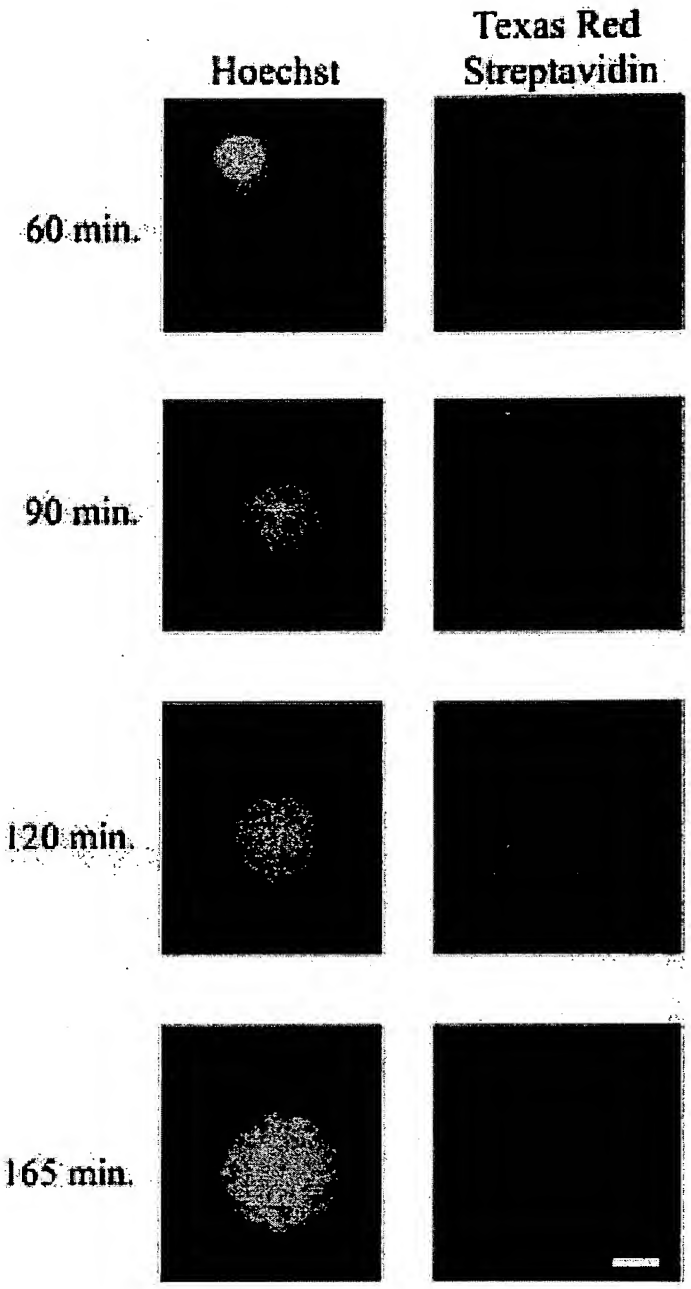

FIG. 5. Morphological changes of human sperm in Interphase-Extract. Sperm nuclei were pretreated, incubated in MII-Extract, and then diluted into 22-min Interphase-Extract containing biotinylated dUTP. Aliquots of sperm were removed at the indicated times. After fixation, samples were stained with Texas Red/streptavidin to detect newly replicated DNA and counterstained with Hoechst dye to visualize total DNA. Nuclei were not stained with Texas Red/streptavidin after 60 min but gradually became larger and increasingly stained thereafter (bar = 11 μm).

synthesis in pretreated human sperm nuclei, a large batch of unfertilized *Xenopus* eggs was synchronously activated at 20°C; groups of eggs were removed after 12, 17, 22, and 26 min and were then used to prepare separate extracts. The results demonstrate that all 4 extracts supported DNA synthesis in human sperm pronuclei, but the extract prepared from eggs incubated for 22 min after activation had the highest DNA synthetic capacity (Fig. 4). These findings were reproduced on 4 separate occasions, and results from 2 of those experiments are shown in Figure 4. The reasons for the difference between the optima for frog erythrocyte and human sperm nuclei remain to be explored but may relate to the fact that sperm chromatin is composed of protamines while erythrocyte chromatin contains histones.

The Kinetics of DNA Synthesis in the Optimized System

Chemically pretreated sperm nuclei incubated in MII-Extract and then diluted into a 22-min Interphase-Extract decondensed, formed pronuclei, and replicated DNA (Fig. 5). The overall rate of DNA synthesis in such reactions was readily measured by [α-^{32}P]dCTP incorporation. The results in Figure 6 (and below) demonstrate that the lag period and rate of DNA synthesis were rather similar in many separate experiments. DNA synthesis ended abruptly and was followed by a plateau period, during which [α-^{32}P]-labeled DNA was recovered as high molecular weight molecules (not shown). Addition of aphidicholin, an inhibitor of DNA polymerase α, blocked subsequent incorporation of [α-^{32}P]dCTP completely (results not shown), indicating that [α-^{32}P]dCTP incorporation was not due to DNA repair.

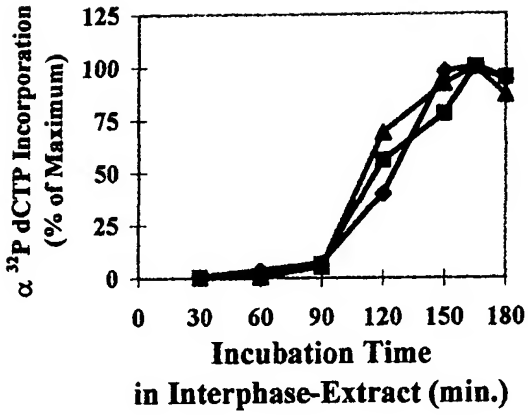

FIG. 6. The rate of [α-^{32}P]dCTP incorporation in the complete optimized in vitro system. Three different aliquots of human sperm from separate proven fertile donors were examined for DNA replication using the optimized 4-step protocol in 3 independently performed experiments. The kinetics of DNA replication are expressed as a percentage of maximum incorporation at the end of S-phase. The similarities in the kinetics of DNA replication and in the lag period before the onset of S-phase among these samples illustrate the reproducibility of the in vitro system.

TABLE 1. The synchrony of DNA replication.

Minutes in interphase extract	Biotinylated-dUTP Incorporation[a]		
	%Not-red nuclei	%Light-red nuclei	%Bright-red nuclei
Experiment 1			
60	99	1	0
165	3	18	79
Experiment 2			
60	98	2	0
165	3	0	97
Experiment 3			
60	100	0	0
165	4	8	88
Experiment 4			
60	98	2	0
165	1	4	95
Experiment 5			
60	99	1	0
165	1	21	78

[a] 50–100 Nuclei per timepoint scored.

Cytological Analysis of the Synchrony of Nuclear Replication

Labeling with [α-^{32}P]dCTP is the most convenient technique for establishing relative rates of DNA synthesis, but it does not reveal the percentage of pretreated nuclei that initiate and proceed through DNA replication. In order to measure this aspect of replication, we employed continuous incorporation of biotinylated dUTP followed by staining with Texas Red/streptavidin (see *Materials and Methods*). Prior to the start of S-phase, all nuclei are small and compact and do not stain red (i.e., do not incorporate biotinylated dUTP). When S-phase begins, nuclei swell and stain light red, indicating that some DNA synthesis has occurred. As S-phase continues, nuclei become larger and redder, in-

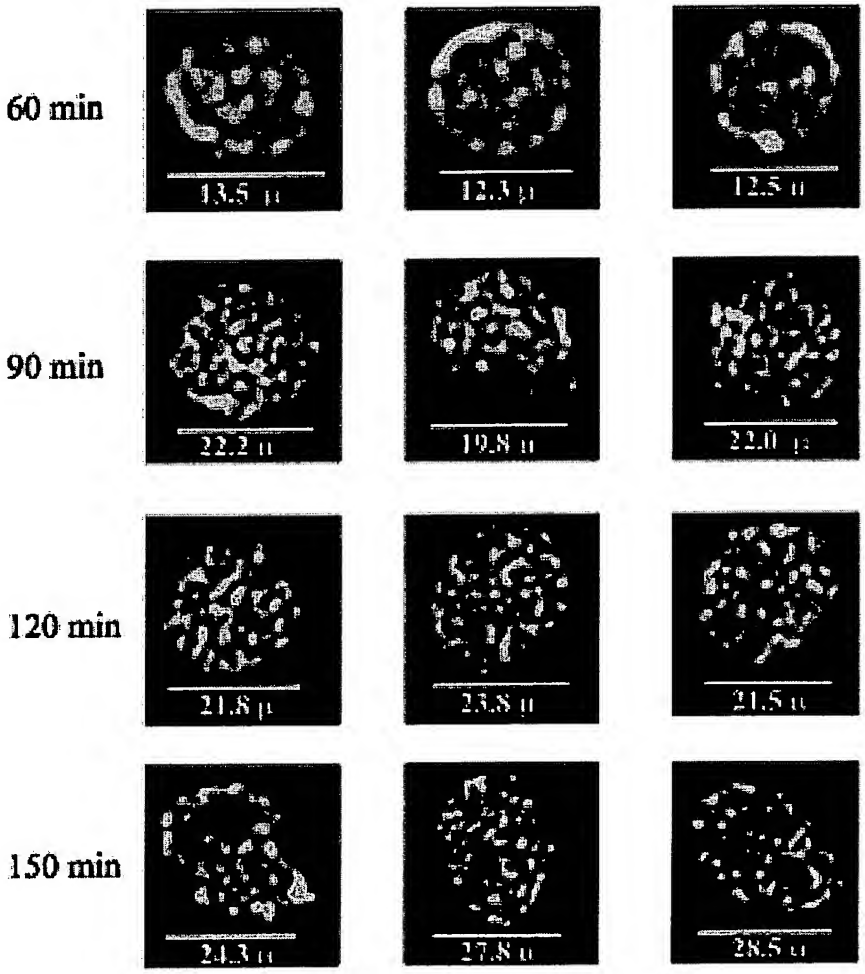

FIG. 7. Deconvolved images of Hoechst-stained replicating nuclei. Individual nuclei were optically sectioned through the Z-dimension, and the resulting images sets were deconvolved using CELLscan imaging software (see *Materials and Methods*). Notice that images are normalized in size. Numbers in each image correspond to measurements of nuclear diameter in the XY-plane. These data demonstrate that the onset of S-phase by 60 min was accompanied by rapid swelling that was followed by more gradual swelling at later times.

dicating that more and more newly synthesized DNA has accumulated in each nucleus (see Fig. 5). Using this approach we scored the percentages of not-red, light-red, and bright-red nuclei in samples collected before and after S-phase. Table 1 displays those data obtained in 5 separate experiments. These results demonstrate that 78–97% of nuclei (average 87%) replicated enough DNA by the end of S-phase to be scored as bright-red. The remaining nuclei, 3–22% (average 13%), were either still unreplicated (not-red) or partially replicated (light-red), even after 165 min.

Deconvolution of Fluorescent Images Also Revealed the Homogeneity of Replicating Nuclei

Conventional fluorescent imaging is useful for assessing relative levels of Texas Red/streptavidin staining among biotin-labeled nuclei, but it does not generate particularly clear images of nuclear structure. This limitation is typical of virtually all fluorescent objects and is attributable to the fact that light emitted from all out-of-focus planes is superimposed on the light emanating from the in-focus plane being examined. Mathematical deconvolution of fluorescent images allows the out-of-focus light to be reassigned back to its plane of origin [19, 20]. Figure 7 illustrates deconvolved images of 12 different human sperm pronuclei during the course of S-phase. Two characteristics of these images stand out: 1) the images demonstrate that the chromatin within decondensing nuclei changed in structure from very thick, ropelike fibers to very thin, threadlike fibers; 2) they show that at each point in time, different nuclei had very similar structures.

Quantitative Analysis of the Extent of Genome Replication via CsCl Labeling

The biotinylated dUTP-labeling experiments described above demonstrate that 78–97% of human sperm pronuclei replicated DNA within 2 h in the optimized system, but they do not rigorously demonstrate the extent of genome replication. Quantitative analysis of total DNA synthesis was carried by labeling replicating DNA with Br-dUTP and then analyzing it via CsCl density gradient centrifugation. Figure 8 reveals that at the start of S-phase (60 min), all of the DNA was unlabeled and was therefore recovered from the light-light (LL) position on the gradient. In contrast, at the end of S-phase (180 min), 21.4% of the total DNA remained unreplicated [LL], while 76.7% had replicated once and was recovered as heavy-light (HL) DNA. An additional 1.9% of the DNA was of intermediate density. The percentage of LL DNA present after 180 min (21.4%) was consistent with the maximum percentage of not-red and light-red nuclei present at the end of S-phase in biotinylated dUTP-labeled samples (22%, see Table 1). This situation may reflect the fact that Br-dUTP partially inhibits DNA synthesis when added at the concentrations needed for extensive substitution of thymidine [10]. Taken together, these results lead us to conclude that biotin-labeled nuclei scored as bright-red by the end of S-phase replicated all or nearly all of each of their genomes.

In order to account for complete genome replication in such a very short S-phase in our in vitro system, virtually all replicons in each nucleus must initiate DNA synthesis at approximately the same time. This conclusion is consistent with Newport's direct measurements of replicon elongation in *Xenopus* sperm nuclei [21] and with classical observations of replicating DNA in early embryonic nuclei [22]. Random shearing of partially replicated DNA mole-

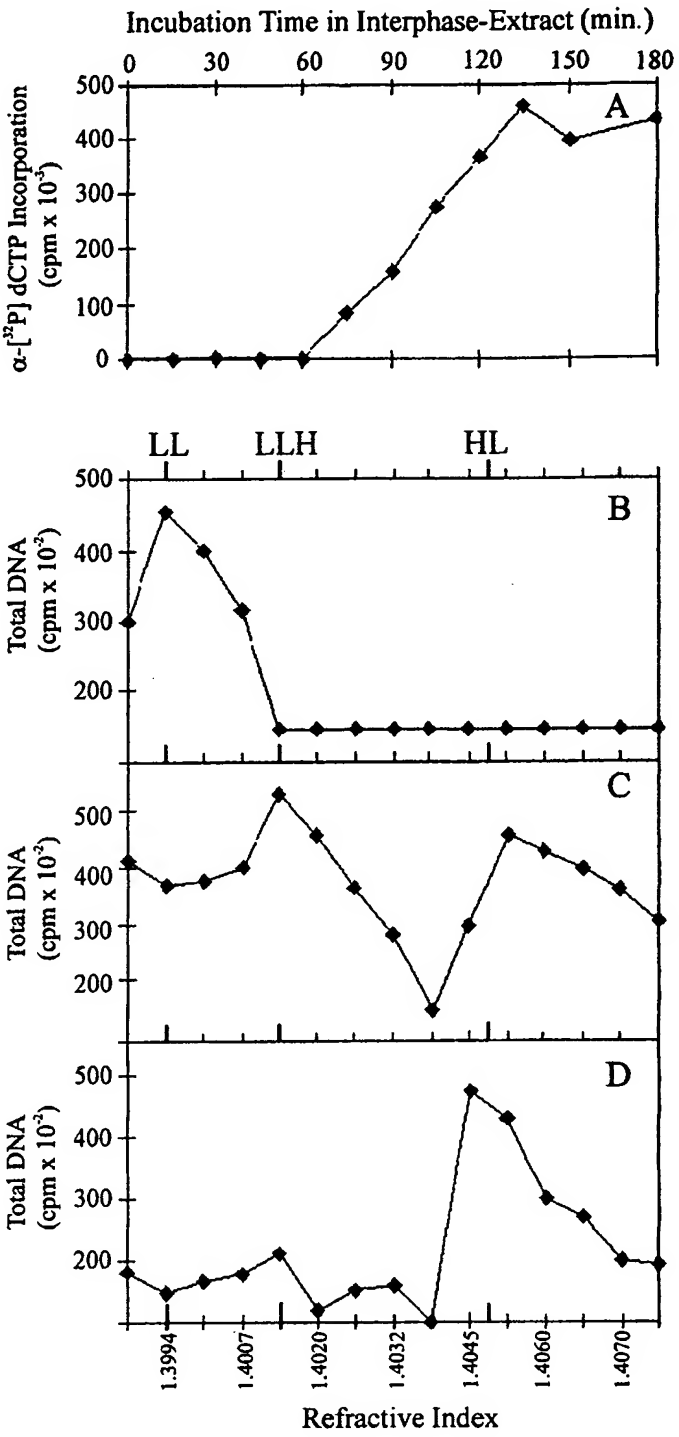

FIG. 8. The extent of genome replication measured by Br-dUTP density labeling. A) Fully pretreated sperm nuclei were incubated in Interphase-Extracts supplemented with [α-^{32}P]dCTP, and aliquots were taken to determine the kinetics of DNA synthesis. **B–D**) The same fully pretreated sperm nuclei were incubated in the same Interphase-Extract supplemented with Br-dUTP instead, and aliquots were taken at various incubation times to assess the extent of replication by CsCl density gradient centrifugation analysis [10]. The incubation times assessed were **B**) 0 min (prior to S-phase); **C**) 120 min (during S-phase); **D**) 180 min (at the end of S-phase). The positions of unreplicated LL DNA (RI = 1.3994), partially replicated LLH DNA (RI = 1.4012), and once-replicated HL DNA (RI = 1.4051) are indicated by heavy tick marks on the x-axes and labeled above panel **B**.

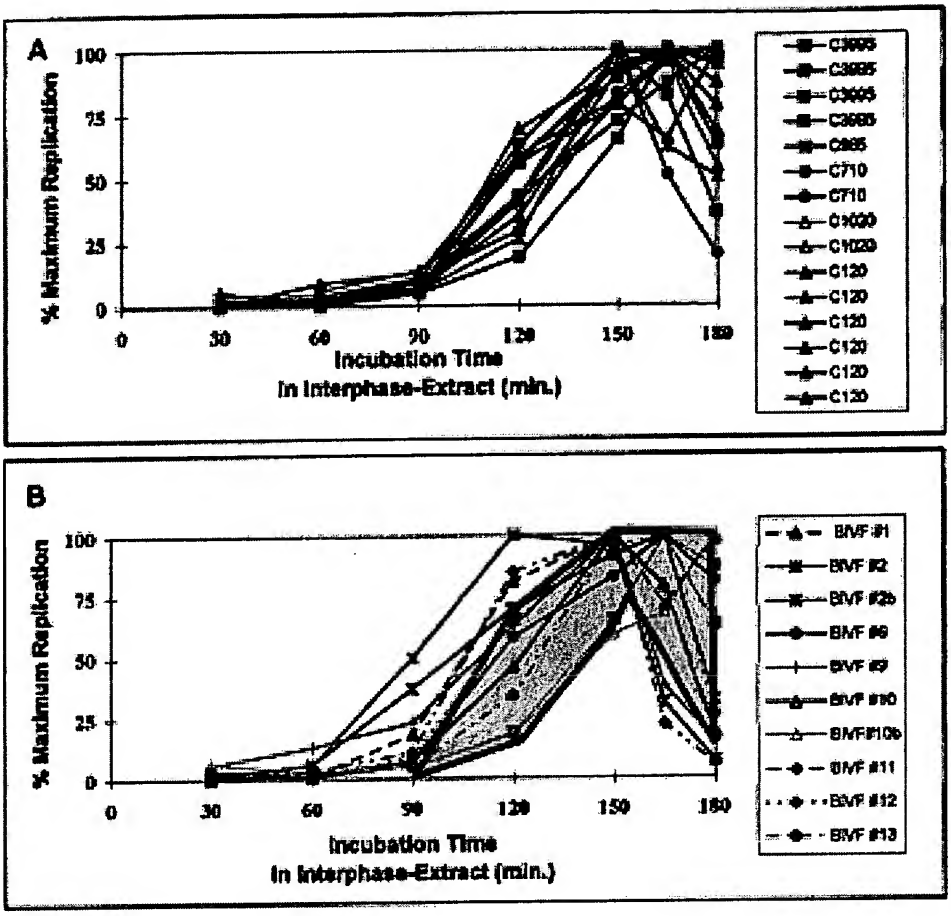

FIG. 9. Use of the *Xenopus* egg extract system to compare the responses of sperm from fertile and subfertile men. A) Kinetics of DNA replication of sperm from proven fertile donors. Each line corresponds to a separate experiment. Fifteen separate experiments using 5 different proven fertile donors are shown. All sperm samples were analyzed using the same extract system. These data establish the range of responses observed in control sperm samples in the egg extract. B) Comparison of kinetics of DNA replication of sperm samples from known fertile and subfertile men. Each line corresponds to a separate experiment. Ten separate experiments using 8 samples from known subfertile patients with 0–3% low normal morphology are shown. The shaded area corresponds to the response range of sperm from proven fertile donors shown in A. Two of the samples from the 8 subfertile donors (BIVF#2 and BIVF#9) displayed rates of [α-^{32}P]dCTP incorporation that were faster than those in control sperm (see text for details).

cules labeled with Br-dUTP would be expected to generate fragments of HL DNA from fully replicated replicons, fragments of LL DNA from unreplicated DNA, and DNA fragments of intermediate density corresponding to transient replication intermediates consisting of unreplicated and replicated DNA (LLH DNA). CsCl density gradient analysis of samples prepared partway through S-phase (120 min in Fig. 8C) demonstrate the presence of both an HL DNA peak and a peak that has a density slightly greater than that of LL DNA. We call this material LLH DNA, and we consistently observed that the LLH peak decreased as the HL DNA increased toward the end of S-phase.

Use of the Xenopus Egg Extract System to Compare the Responses of Sperm from Fertile and Subfertile Men

To assess the diagnostic potential of our *Xenopus* egg extract system, we have started to compare the responses of sperm samples from known fertile and subfertile patients. Thus far samples have been prepared from 10 subfertile men undergoing fertility treatment at the Boston Fertility Laboratories, Inc. All samples scored in the 0–3% range for normal morphology. Control samples were obtained from 5 proven fertile donors displaying 6–14% normal sperm morphology.

Regardless of the fertility status of each donor, all sperm samples underwent nuclear envelope breakdown and chromatin reorganization in the MII-Extract and went on to acquire envelopes, form pronuclei, and undergo DNA replication upon dilution into the Interphase-Extract. Analysis of the kinetics of sperm DNA replication from 15 separate experiments using 5 proven fertile donors established the range of responses that could be observed in normal sperm samples (Fig. 9A). Two of the samples from the 8 subfertile patients displayed rates of [α-^{32}P]dCTP incorporation that were faster than any of those observed for the proven fertile donors. These differences in replication rates were most evident after 90 min, just after DNA synthesis had begun. Fluorescent microscopy did not reveal why the 2 subfertile samples synthesized DNA more rapidly (data not shown).

DISCUSSION

We have designed and optimized a 4-step protocol for in vitro reactivation and replication of human sperm that recapitulates the process of pronucleus development in fertilized mammalian eggs. In Step 1, intact sperm are treated with lysolecithin to permeabilize the plasma membrane. This step replaces sperm-egg fusion during normal fertilization. In Step 2, permeabilized sperm are incubated in dithiothreitol, a reducing agent that disrupts the disulfide bonds between the protamines of the sperm head. During normal mammalian fertilization this step takes place within the egg cytoplasm in response to glutathione. In Step 3, chemically pretreated sperm are incubated in MII-Extract prepared from unfertilized/unactivated *Xenopus* eggs arrested in meiotic metaphase II. In the presence of the MII-Extract, pretreated sperm undergo nuclear envelope breakdown and chromatin reorganization and eventually disperse into separate chromosomes. The MII-Extract mimics the environment that the sperm encounters immediately after entering the egg's cytoplasm. In Step 4, pretreated sperm nuclei are diluted into an Interphase-Extract prepared from *Xenopus* eggs that have been induced to enter the cell cycle

in response to Ca^{2+} ionophore treatment. The Interphase-Extract mimics the environment of an activated egg and has been optimized for DNA replication in human sperm pronuclei. Under these conditions, all nuclei form pronuclei, and 78–97% of these pronuclei initiate and complete genome duplication. Replication of each human genome takes less than 2 h in this system, consistent with the very fast cell cycle of the intact frog egg [23]. Our results are in accord with a substantial body of evidence demonstrating that factors in metaphase II-arrested egg cytoplasm are required for subsequent reinitiation of DNA synthesis in activated egg cytoplasm [24]. The use of 2 types of *Xenopus* egg extracts in our 4-step procedure distinguishes this work from previous studies of human sperm nuclear reactivation in vitro.

To our knowledge this is the first fully optimized system for reactivation and replication of human sperm nuclei in *Xenopus* egg extracts. Xu et al. [8] recently reported complete human sperm nuclear replication in a system employing only a single extract prepared from activated *Xenopus* eggs. However, DNA synthesis was only 60% complete after 4 h and required 9 h to achieve genome doubling. Fertilized *Xenopus* eggs divide approximately 12 times and synthesize at least 1000 genomes worth of DNA in that amount of time. Although we have not directly compared our system to that of Xu et al. [8], our results suggest that the rate of DNA synthesis is greatly increased by preincubation of sperm nuclei in MII-Extract prior to dilution into Interphase-Extract. We further propose that the reason Xu et al. [8] observed DNA replication at all is that they prepared their extract from metaphase-II-arrested eggs that were activated at the time of extract preparation. Extracts prepared under these conditions contain transient levels of H1 kinase activity.

We foresee that in vitro systems for human sperm pronuclear formation and replication will prove useful for a variety of future studies. For instance, we are currently using fluorescent in situ hybridization to analyze the location and unfolding of centromeres and telomeres in decondensing sperm nuclei. Our results confirm that most centromeres co-localize in a chromocenter in the condensed sperm genome [25], and they demonstrate that the chromocenter persists during the initial decondensation process in MII-Extract (personal communication with Serra et al.).

We are also utilizing our optimized system to study the properties of sperm from normal and subfertile men. Preliminary comparisons of the responses of known fertile and subfertile sperm in our optimized system show no correlation between low percentage normal morphology and the ability of sperm from subfertile men to form pronuclei and replicate in vitro. However, relative to proven fertile sperm, 2 of 8 subfertile sperm samples exhibited slightly faster-than-normal kinetics of DNA replication. Further analysis will be required to determine whether differences in the kinetics of pronuclear DNA synthesis can be used to distinguish fertile from subfertile sperm samples and whether a heterologous system (*Xenopus* egg extract) can produce data that are representative of a homologous system (human eggs).

Brown and coworkers [26] have reported that a significant percentage of sperm samples prepared from infertile men undergo abnormal nuclear swelling and DNA synthesis in an egg extract assay, while virtually no control samples from fertile men fail the same assays. These investigators use a single extract prepared from activated eggs, as compared to our two-extract system. It is possible that we have not observed major differences in sperm nuclear decondensation and replication among samples from infertile men because of the high efficiency of our two-extract system. Unlike our experimental system, the one-extract system described by Brown and coworkers [26–28] provides no evidence for complete genome replication, even among control sperm samples. As we have tried to emphasize and demonstrate in this paper, mere detection of nuclear swelling, or incorporation of a radioactive precursor, does not demonstrate whether DNA synthesis is efficient, extensive, or synchronous.

Finally, we suggest that our in vitro system may provide insights into the critical events that must take place within the human egg after ICSI to achieve normal development. For instance, we have observed that the length of time that a sperm nucleus is incubated in the MII-Extract greatly affects the rate and extent of DNA replication in the subsequent S-phase. Maximum replication occurs when maximum nuclear swelling takes place prior to S-phase; but fully swelled nuclei gradually become more compact if they remain in the MII-phase cytoplasm too long, and they no longer replicate efficiently. These phenomena warrant detailed biochemical investigation in light of reports that eggs fertilized by ICSI show slower-than-normal rates of cell cycle activation [29] and delayed patterns of nuclear decondensation in monkeys [30].

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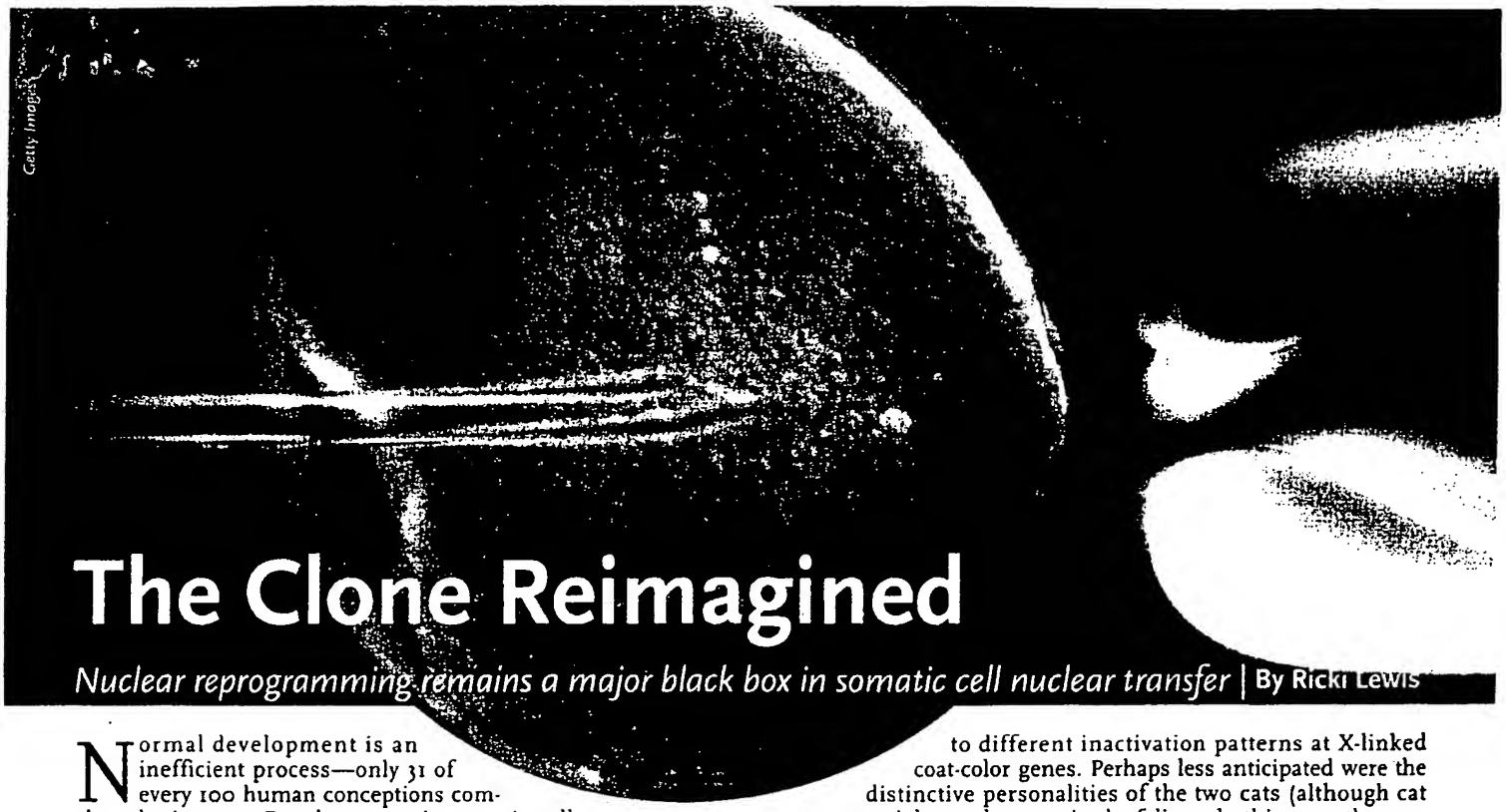


The Clone Reimagined

Nuclear reprogramming remains a major black box in somatic cell nuclear transfer | By Ricki Lewis

Normal development is an inefficient process—only 31 of every 100 human conceptions complete the journey. Development via somatic cell nuclear transfer (SCNT), under the direction of adult DNA from a specialized cell is even less efficient, with success rates of about one in 300 or worse. Although nearly as many questions remain as when SCNT was first attempted half a century ago, researchers are closing in on why it is so difficult to emulate normal development starting with this most unusual genetic headquarters.

The major distinction between development via sperm and egg versus SCNT is a profound perturbation of gene expression. The deviations arise from various sources, including influences of the culture medium and factors in the nucleoplasm and cytoplasm. When all goes well, morphogens interact to effectively reprogram the new nucleus, which is vital to resetting the developmental program. But even the most prominent researchers readily admit that much of what transpires in SCNT is a mystery. Says John Gearhart, director of stem cell biology at the Institute for Cell Engineering at Johns Hopkins University, "If you figure out how to reprogram a nucleus, let me know."

MORE THAN NEW DNA Biologists know some steps in the basic choreography of fertilization. In most mammals examined, just before the male and female pronuclei meet and merge, the paternal genome is globally and actively demethylated, and the maternal genome passively follows. About when the blastocyst implants in the uterine wall, the first inklings of determination and then differentiation begin as methylation ensues in a stepwise, lineage-specific manner. This epigenetic pattern is stamped on further cell generations. Imprinted genes escape the initial CH_3 removal, remaining tenaciously methylated at key stretches of CpG dinucleotides. For these genes, only one parent's allele is normally expressed in offspring.

"The goal in nuclear transfer (NT) is to reproduce the conditions of a very early, one-cell embryo produced by fertilization, because these embryos clearly develop very nicely," says Tony Perry, head of the laboratory of mammalian molecular embryology at the Riken Center for Developmental Biology in Kobe, Japan. But SCNT is fraught with difficulties, both in technique and assumptions. Even the seemingly logical assumption that clones would be identical rarely holds up.

"Animal clones may have greater variability in some traits than conceiving the old-fashioned way," says Randy Jirtle, a professor of radiation oncology at Duke University Medical Center. Cloned cat CC, for example, differs from her nucleus donor in coat color due to different inactivation patterns at X-linked coat-color genes. Perhaps less anticipated were the distinctive personalities of the two cats (although cat owners might not be surprised—felines do things as they see fit, even accessing their genomes). And pigs cloned at Texas A&M University vary significantly in levels of certain blood components.[1]

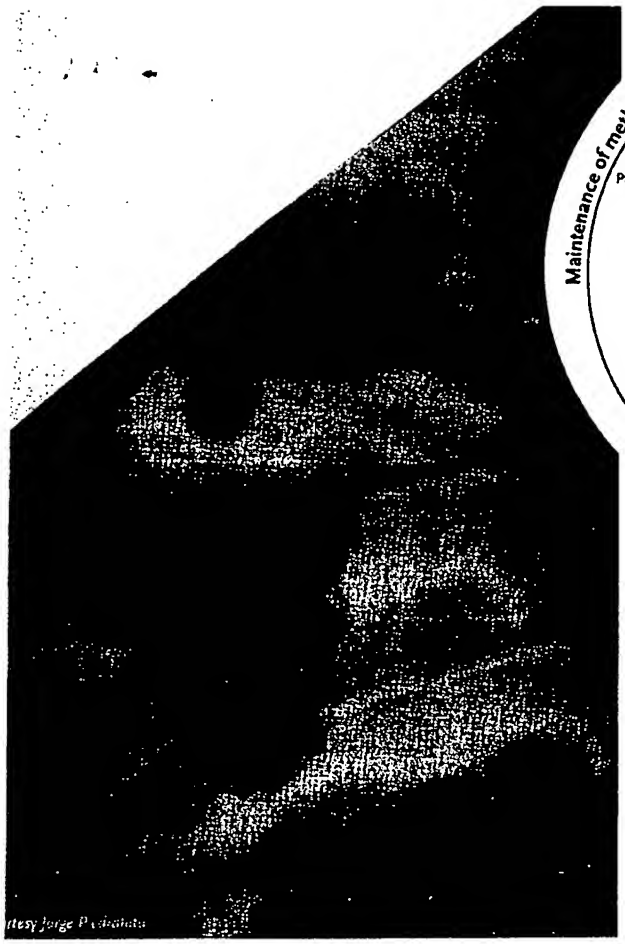
FIFTY YEARS OF NUCLEAR TRANSFER CC and the Texas porkers are members of a select club: the successfully cloned. In 1952, Robert Briggs and Thomas J. King first transferred nuclei from somatic cells to enucleated eggs and watched development unfold in the northern leopard frog *Rana pipiens*. In the 1960s and 1970s, SCNT experiments continued in the South African clawed frog, *Xenopus laevis*, by John Gurdon and others. Decades later Dolly the sheep, Cumulina the mouse, and cloned calves Charlie and George brought mammals into the society, demonstrating that SCNT can succeed—albeit rarely and problematically.

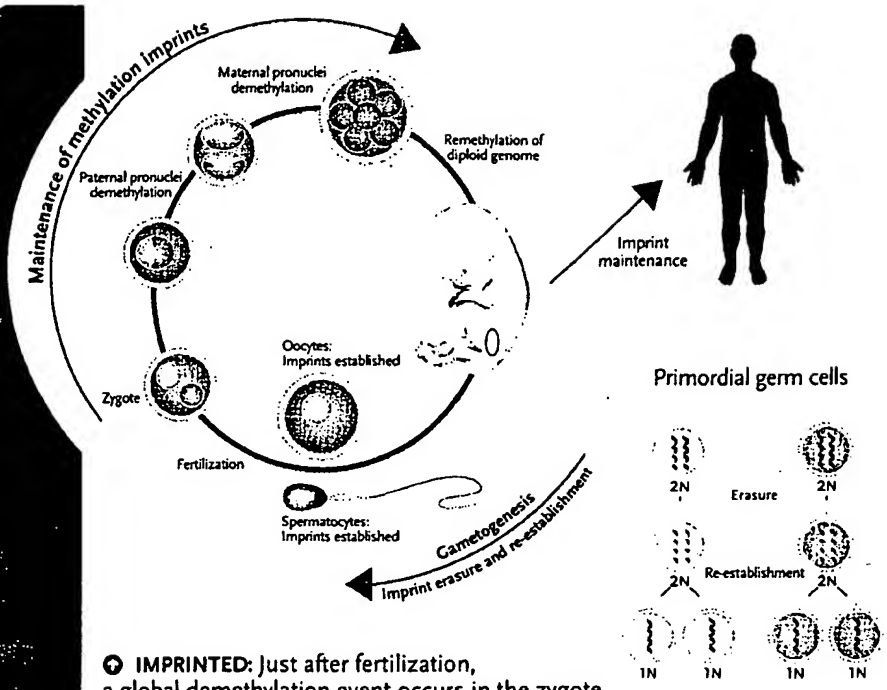
"Briggs, King, and Gurdon were an excellent starting point for a student of nuclear transfer. The problem is that they are also something of a finishing point," says Perry. Little has been learned since then about what happens when SCNT works. What is known, generally, is that nuclear reprogramming is the first major hurdle a cell created by SCNT must surmount. In successful cloning experiments, the problem appears to work itself out, although whether development unfolds normally isn't known. A relocated somatic cell's genome must silence those genes that made it part of a breast or connective tissue, while activating the genetic program that normally runs in a fertilized ovum.

The basic problem: A somatic cell's DNA in an egg is a decidedly unnatural circumstance. Eclectic experiments are revealing some of the points at which the unusual embryonic journey of SCNT goes awry.

CULTURE SHOCK AND TRANSFER WOES Culture medium that nurtures donor cells can affect later gene expression if it contains nutrients that methylate DNA. This connection became evident rather unexpectedly when assisted reproductive technology registries began reporting cases of extremely rare human imprinting disorders, such as Beckwith-Wiedemann syndrome, Angelman syndrome, and retinoblastoma, associated with in vitro fertilization (IVF).[2] Although the higher incidence could reflect reporting bias or underlying infertility issues, another explanation is that the culture medium methylates certain imprinted genes in cells destined for IVF. Jirtle's group has shown that single carbons from methionine and choline form the basis of methyl groups, and folic acid, vitamin B_{12}, and pyridoxal phosphate are cofactors essential for methyl metabolism.[3]

If methionine in culture medium causes imprinting disorders in children born after IVF, it might also alter methylation of DNA





IMPRINTED: Just after fertilization, a global demethylation event occurs in the zygote, first in the paternal pronucleus, followed by the maternal pronucleus. Imprinting established during gametogenesis must resist this demethylation process. Remethylation of the diploid genome occurs post-implantation and sets secondary imprints that are maintained for the life of the individual. (From S.K. Murphy, R.L. Jirtle, *BioEssays*, 25: 577–88, 2003.)

THESE LITTLE PIGGIES: The cloned pigs show similar, yet distinct spotting patterns due to epigenetic influences. The migration of pigmented cells is affected by random phenomena.

destined for SCNT. "We must learn how to optimize the media to maintain epigenetic profiles," says Jirtle.

Rudolf Jaenisch, professor of biology at the Massachusetts Institute of Technology, and colleagues investigated an effect of media conditions by comparing expression of 10,000 genes in the livers and placentas of mice cloned from "stale" cultured embryonic stem cell (ESC) nuclei or "fresh" cumulus cell nuclei.[4] All the mice had large offspring syndrome (LOS), which affects most cloned mammals as well as those derived from assisted reproductive technologies. The researchers found double the normal expression for 286 genes from cumulus-cell clones and 221 genes from ESC clones, with 76% overlap. The affected genes encode proteins essential for embryogenesis, including growth factors, receptors, enzymes, clotting factors, MHC antigens, and histone deacetylases. But determining whether or not these gene-expression profiles were the result of culture conditions required further experiments.

Using a manipulation called tetraploid complementation[5] they isolated an effect of culture conditions. This technique overcomes a major limitation of cloning from ESCs—they cannot create a placenta. Normally, explains Jaenisch, ESCs are injected into a diploid blastocyst, and the resulting mosaics must be bred to obtain a clonal animal.

Using tetraploid helpers generates ESC-derived embryos faster, Jaenisch says. "Two cells fuse to form a tetraploid cell that divides to form a very nice blastocyst, but it doesn't develop. The tetraploid cells exclude the epiblast lineage, so they don't make an embryo." But mix embryonic stem cells and tetraploid cells, "and we get a mouse embryo that is entirely derived from ES cells, but supported by a tetraploid placenta." Such an embryo is a clone of the embryonic stem cell, but it is not necessarily derived using SCNT, Perry points out.

Unlike NT-derived mice using ESC or cumulus cell nuclei, the mice whose development began with the help of tetraploid extraembryonic structures had normal placentas and no LOS. Expression of some genes, however, differed in the NT ESC-derived mice and the tetraploid-derived mice, revealing effects on the ESC clones caused by NT.

But the observation that the expression of some genes was altered in embryos derived from the two sources of ESCs (NT and tetraploid complementation), but not in cumulus-derived embryos, suggests that some of the differences in gene expression in a clone reflect nucleus source.

THE CENTER OF IT ALL The amphibian work of the 1950s and 1960s revealed that the younger or less specialized the donor nucleus, the higher the success rate of SCNT. Results in mice are similar: Transferring nuclei from 2-celled embryos leads to a 22% birth rate, yet that number plummets to 14% for a 4-cell-stage donor and to 8% for an 8-cell-stage donor.[6] Only 1–3% of nuclei transferred from differentiated cells from adult frogs support full prenatal development. Using donor cells functionally related to the germline increases those odds slightly.

But even the most specialized cell does not have its genetic headquarters irreversibly silenced. Groups at Harvard University and Rockefeller University cloned mice from highly specialized olfactory sensory neurons (OSN).[7,8] Each OSN expresses only one of roughly 1,500 olfactory receptor genes, but clones had the full olfactory repertoire indicating donor-nucleus reprogramming.

The list of adult cell types whose nuclei can support at least limited development is growing. Jaenisch and Konrad Hochedlinger had previously cloned mice using a distinctive adult somatic cell nucleus donor—B cells, which normally rearrange their genomes in response to exposure to antigen. Every cell in the cloned mice had the altered genome characteristic of the donor B cell.[9] Even cancer-cell nuclei, in mice, can recapitulate very early development. Jaenisch's group used DNA from leukemia, lymphoma, and breast cancer cells to generate preimplantation embryos,[10] and James Morgan's group at St. Jude Children's Research Hospital in Memphis, Tenn., used nuclei from medulloblastoma cells.[11]

If SCNT works with a donor cell so specialized it is dubbed "terminally differentiated"—as with an OSN—then anything is possible, given the appropriate signals. And that's where the nucleoplasm and ooplasm enter the picture.

JUMPSTARTING THE PROGRAM Once a somatic cell nucleus is transferred, factors in the recipient ooplasm stimulate reprogramming. At this point, the egg cells—technically still oocytes before fertilization—have had their DNA removed and are arrested at metaphase of the second meiotic division, when the nuclear membrane temporarily breaks down. "Metaphase II chromosomes are removed and not nuclei. The question is if and which crucial nuclear proteins are left behind," says Hans Schöler, director of the Max Planck Institute for Molecular Biomedicine in Munster, Germany.

Schöler and Jeong Tae Do, both formerly at the Center for Animal Transgenesis and Germ Cell Research at the University of Pennsylvania, demonstrated a role for nucleoplasm by fusing ESCs and their derivatives with neurosphere cells (NSCs), as a model of induced dedifferentiation that is easier to work with than oocytes.[12] Fusing an NSC with an entire ESC activates *Oct4*, a gene expressed in pluripotent cells of the embryo up until gastrulation, which is not normally expressed in NSCs, and therefore marks reacquired pluripotency. The researchers then separated ESCs into karyoplasts (membrane-bounded nuclei with a hint of cytoplasm) and cytoplasts (membrane-bounded cytoplasm). NSCs fused with ESC karyoplasts turned on *Oct4*, but NSCs fused with ESC cytoplasts did not. Therefore, the ESC nucleus contains factors necessary for reprogramming. Further, when the researchers blocked DNA replication by treating the ESC karyoplasts with mitomycin C, *Oct4* was still expressed—indicating that something in the nucleus other than DNA plays a role in reprogramming.

Just as important as turning on genes such as *Oct4* is turning off the genes that sculpted characteristics of the donor cell. John Gurdon and Ray Ng at the University of Cambridge showed that cells of cloned *Xenopus* "remember" their origin and express genes normally active in the nucleus donor cell.[13] When the researchers transferred nuclei from neuroectoderm into enucleated oocytes, some of the resulting clones not only overexpressed the neuroectoderm marker *Sox2*, but did so in their endoderm, indicating that the inappropriate gene expression was both out of time and out of place.

Skewed expression of *Oct4* and *Sox2* are but two ways that SCNT-derived offspring veer from normalcy. If aberrant gene expression ensues and persists whenever a somatic cell nucleus finds itself in ooplasm—as Jaenisch's 10,000-gene arrays suggest—it isn't surprising that SCNT leading to birth is exceedingly rare.

Taken together, experiments that correlate unusual gene expression patterns with the events of NT are revealing many points at which development leaves its normal path—suggesting that a clone such as Dolly may have superficially been just a sheep, but her tissues may have told a different story. It is perhaps ironic that as politicians and the public continue to debate SCNT in tissue engineering, and to universally condemn its use for human reproductive cloning, developmental biologists are discovering that in reproduction, the ends do not necessarily reflect the means.

Kevin Eggan, a junior fellow in the department of molecular and cellular biology at Harvard University, who was involved in cloning a mouse from an olfactory neuron last year, sums up, "Everything we know from animal experiments suggests that a cloned child would likely be in danger of a number of developmental abnormalities, both known and unknown." ⊗

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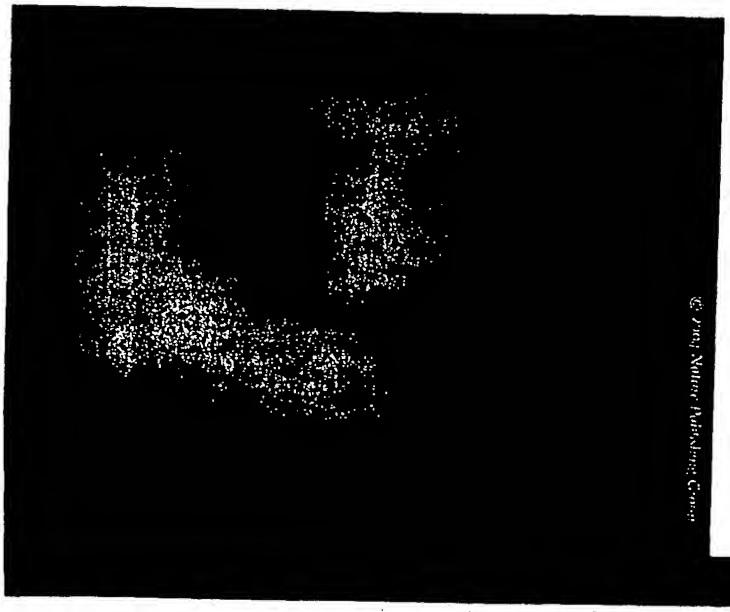

GLOWING GOOD RESULTS: A mouse derived from a mature olfactory sensory neuron nucleus contained the complete olfactory repertoire. This mouse was created using tetraploid complementation from an OSN3 embryonic stem cell line expressing green fluorescent protein. (From K. Eggan et al., *Nature*, 428:44–9, 2004.)

Cloned rabbits produced by nuclear transfer from adult somatic cells

Patrick Chesné[1], Pierre G. Adenot[1], Céline Viglietta[1], Michel Baratte[2], Laurent Boulanger[1], and Jean-Paul Renard[1*]

We have developed a method to produce live somatic clones in the rabbit, one of the mammalian species considered up to now as difficult to clone. To do so, we have modified current cloning protocols proven successful in other species by taking into account both the rapid kinetics of the cell cycle of rabbit embryos and the narrow window of time for their implantation after transfer into foster recipients. Although our method still has a low level of efficiency, it has produced several clones now proven to be fertile. Our work indicates that cloning can probably be carried out successfully in any mammalian species by taking into account physiological features of their oocytes and embryos. Our results will contribute to extending the use of rabbit models for biomedical research.

The rabbit is gaining attention in biotechnology because it offers several advantages over other laboratory animals for the study of several human physiological disorders[1,2]. Not only can physiological manipulations in this species be more easily carried out than in mice because of its larger size, but it is also phylogenetically closer to primates than are rodents[3]. Currently, the use of rabbits is limited to large-scale production of foreign proteins[4]. Thus cloning, associated with the genetic modifications of donor cells, would greatly enhance the possible use of this species in biotechnology. In contrast to several other mammalian species, however, the rabbit has not been very amenable to somatic cloning[5,6], despite its pioneer role in defining nuclear transfer (NT) methods in mammals[7]. Here we describe a method that has allowed us to produce several healthy and fertile somatic clones of rabbit at about the same frequency as other mammalian species. Our results will contribute to extending the use of rabbit models for biotechnological applications.

Results and discussion

Rabbit NT embryos were reconstructed by electrofusion of freshly collected cumulus cells with recipient enucleated metaphase II (MII) ooplasm. We chose this type of nuclear donor cells because they had been initially used as models to demonstrate the feasibility of somatic cloning[8,9]. Confocal observations of reconstructed embryos, fixed 1 h after electrofusion, showed that donor nuclei exposed to MII ooplasm had condensed into chromosomes[10] (Fig. 1A). Instead of orderly chromosome arrays typical of MII oocytes, we observed misaligned metaphase plates, very similar to those previously shown in the mouse to be compatible with full-term development[8]. We therefore activated NT embryos through a second set of electrostimulation and incubated them in the presence of cycloheximide (CHX; a protein synthesis inhibitor) and 6-dimethylaminopurine (6-DMAP; a kinase inhibitor), two drugs known to facilitate the exit from artificially activated MII stage, but with potential detrimental side effects on the cell cycle[11,12]. Because the rabbit zygote enters S phase very early after activation[13], we focused on reducing the time of exposure to these inhibitors. We observed that they accelerated pronuclear formation in artificially activated rabbit oocytes, but also caused high rates of parthenogenetic development to the blastocyst stage (90%, $n = 130$), even when the time of incubation was reduced to 1 h. Upon removal of the inhibitors, 72% ($n = 25$) of NT embryos exhibited interphasic structures (Fig. 1B), and 1 h later all were in interphase (Fig. 1C). When left in culture, 47% ($n = 135$) developed into blastocysts at day 3 (D3). Their growth as determined from cell number counts at D3 and D4 was slower than that of blastocysts derived from *in vivo* or *in vitro* zygotes. At D4, the number of cells in NT blastocysts was similar to that of *in vivo* or *in vitro* zygotes at D3 (Fig. 2A).

In rabbits, a rapid and significant expansion of blastocysts occurs *in vivo* and stretches the surrounding walls of the uterus so that their individual positions on the uterine horns become easily recognizable as "implantation sites" as early as D6 (ref. 14). Implantation, however, starts only at D7.5, with the progressive dissolution of blastocyst coverings apposed during the transit of the embryo through the female genital tract, thereby contributing to the narrow window of implantation in this species. Upon dissection of the uterine horns at D8, we found that NT embryos could form some implantation sites (7 out of the 91 embryos transferred, 7.7%) following their transfer into synchronous recipients mated with a vasectomized male at the same time as donor females were mated. However, no embryonic structures were seen. Transfer into asynchronous recipients mated 16 h after the donor females were mated resulted in an increase in the implantation rate, which became only slightly lower than controls (12/59 or 20.3%, and 15/54 or 27.8%, respectively). Under these conditions we could recover embryos at the advanced blastocyst stage (see photo in Fig. 2B), but still surrounded by thin coverings of extracellular material, and thus, equivalent to D7 normal embryos[14]. None of the recipients transplanted either synchronously or asynchronously (−16 h) could be diagnosed pregnant at midgestation (Table 1), even when co-transferred with unmanipu-

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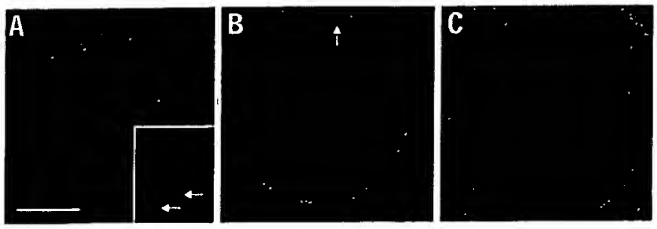

Figure 1. Confocal images of unicellular NT embryos immunolabeled with the anti-α-tubulin antibody (green) and DNA stained with propidium iodide (red). (A) Before the second set of electrostimulation, a misaligned metaphase plate was associated with the spindle, and sometimes individual chromosomes (arrows in the insert) were localized near the spindle poles (insert: 3-fold magnified view of the spindle region). (B) Upon removal of CHX and 6-DMAP, 72% of NT embryos (n = 25) showed a small nucleus and an interphasic microtubular network (arrow). (C) One hour after removal of the drugs, all NT embryos were in interphase and 71% (n = 17) exhibited a single and large pronucleus-like nucleus like those observed in normal rabbit zygotes (not shown). Bar, 50 μm.

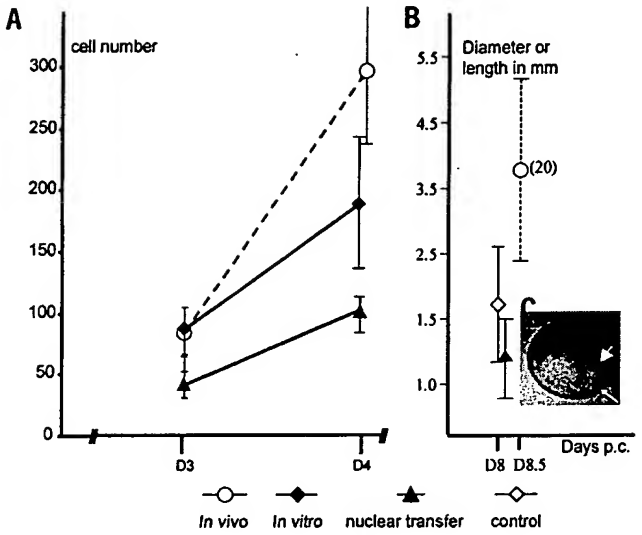

Figure 2. Development of rabbit blastocysts reconstructed with cumulus cells or derived from *in vivo*–fertilized embryos. (A) *In vitro* increase in cell number (mean ± s.e.) between D3 and D4 of embryos either recovered directly from donors (*in vivo*, n = 27), or cultured from the one-cell stage (~20 h post-hCG) after either natural mating (*in vitro*, n = 44) or nuclear transfer (NT, n = 31). (B) Mean diameters or lengths (in mm ; ± s.e.) of the embryonic disks of D8 blastocysts recovered directly from donors (*in vivo*)[20], following transfer into recipients at the one-cell stage after natural mating (control, n = 9), or resulting from NT (n = 7). (C) Example of a retarded NT blastocyst recovered at D8 after transfer at the four-cell stage into a –16 h asynchronous recipient; embryonic disk (large arrow) is visible but the blastocyst is still surrounded by a thin layer of embryo covering (small arrow) that should normally have disappeared at D7 (ref. 14).

lated "helper" embryos of another strain (Fauve-de-Bourgogne), or transferred with an excess of NT embryos (up to 39 per female; data not shown). These observations suggested that only very few NT blastocysts could implant because their development was too delayed. In one case, we could observe a D8 NT blastocyst already adhering to the uterine epithelium and very similar in size to normal implantation controls. We therefore extended the asynchrony between donor and recipient females from 16 to 22 h. Such a marked asynchrony at early cleavage stages of development had not been attempted previously with NT embryos, but can be compatible with full-term development of fertilized eggs[15,16]. Under these conditions, 10 out of 27 (37%) of the –22 h asynchronized recipients were diagnosed pregnant after palpation at D14. Four of these gave birth at D31 to six live kits (Fig. 3) weighing 30–90 g (mean value, 65 g). Such variability is also observed with kits born from reduced litter sizes (one to four fetuses) occasionally obtained in our facilities. Expression of a green fluorescent protein (GFP) transgenic marker from hair follicles (Fig. 3) and from lymphocytes (not shown) confirmed that kits resulted from NT of cumulus cells. Two kits of normal morphological appearance (respective weights 90 and 30 g) died one day after birth, for one of whom we suspect failure in the adoptive process from the lactating mother. The four others are developing normally, and two of them (Fig. 3, B1), when tested for fertility by natural mating, gave birth to seven and eight healthy kits, respectively.

In conclusion, our results show that the former limitations to successful rabbit somatic cloning have been overcome by taking into account species differences in oocyte physiology and early embryonic development. Both a shortened timing for otherwise classical activation procedures and the transfer of reconstructed embryos into recipients retarded by nearly one day had a decisive influence on the *in vivo* development of NT embryos. The maximization of the developmental response of rabbit oocytes to external activating stimuli, through controlled Ca^{2+} stimulation regimes[17] and characterization of the embryonic signals that regulate rabbit uterine epithelial responsiveness at implantation[18], should help to improve term survival rates of embryos reconstructed with different types of somatic and cultured cells.

Table 1. *In vivo* development of rabbit somatic nuclear transfer embryos

Type of recipients	Synchronous	Asynchronous (–16 h)	Asynchronous (–22 h)
Stage of embryos	1-cell	1-cell	4-cell
No. of reconstructed embryos	554	523	775
[No. of replicates]	[19]	[18]	[27]
No. of fused embryos	427	346	612
[% from reconstructed]	[77.1]	[66.2]	[79.0]
Total transferred	367	346	371
[% from fused]	[100.0]	[100.0]	[60.6]
No. of recipients transferred	19	18	27
No. of recipients pregnant at day 14	0	0	10
[% from transferred]			[37.0]
No. of recipients delivering	0	0	4[a]
[% from transferred]			[14.8]
No. of kits born			6
[% from embryos transferred]			[1.6]
Alive at weaning			4
Mean weight of kits at birth (g)			65 ± 20[b]

[a]Abortions between day 15 and day 29 of pregnancy (13 cotyledons and degenerated fetuses recovered).
[b]Mean weight of kits at birth in our facilities: 55.8 ± 17.0 g (sample size, n = 51).

Experimental protocol

Source of oocytes and cumulus cells. MII oocytes were collected from superovulated does of New-Zealand breed 16 h after human chorionic gonadotrophin (hCG) injection and mating to a vasectomized male. They were incubated in 0.5% hyaluronidase (catalog no. H3506; Sigma, St. Louis, MO) for 15 min to remove cumulus cells by gentle pipetting. For nuclear transfer, oocytes were enucleated as described[19]. All manipulations were done in M199 (Life Technologies, Rockville, MD), 20 mM HEPES (Sigma), supplemented with 10% vol/vol FCS (Life Technologies).

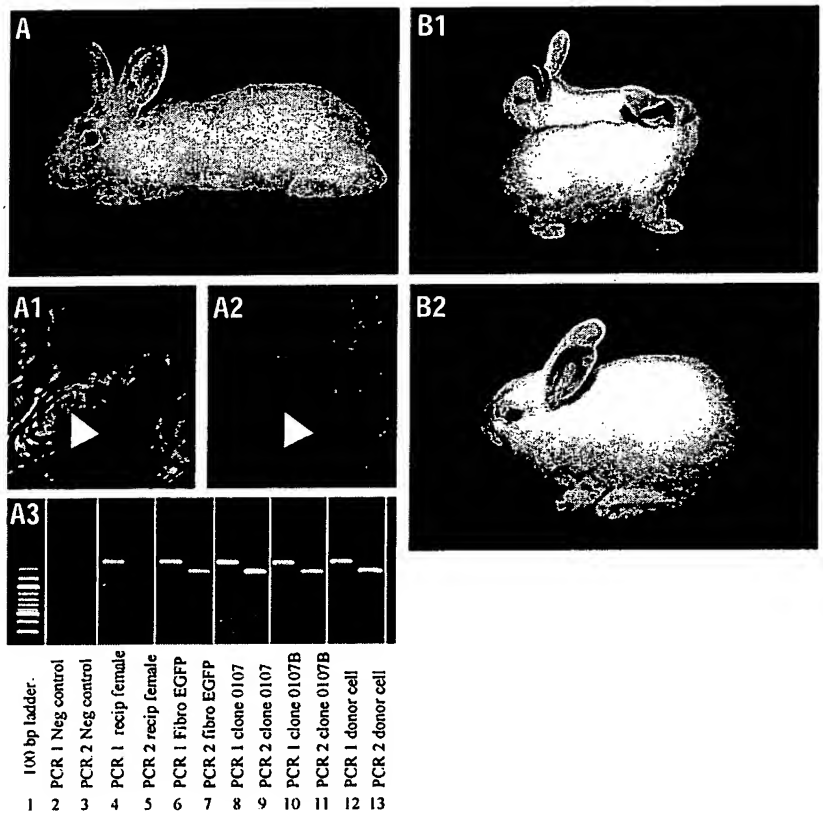

Figure 3. Rabbits born from somatic nuclear transfer. (A) Cloned rabbit 0107 with corresponding controls: (A1) expression of the EGFP protein fluorescence (arrowhead) detected by confocal microscopy from hair follicles obtained from an ear biopsy at 1 month of age; (A2) the same under transmission light; (A3) amplifications of the *EGFP* transgene (PCR 2) and of the exon 10 of the *CFTR* gene used as DNA quality control (PCR 1) with expected fragment sizes of 240 bp for the *CFTR* gene and 350 bp for the *EGFP* transgene. This confirms that rabbit 0107 and its littermate 107b (who died 1 day after birth) were derived from the donor cumulus cell. (B1, B2) Three other rabbits from two different litters; rabbits in B1 have now proved to be fertile.

Contemporary cumulus cells were obtained from the New Zealand breed or F1 New Zealand × Fauve-de-Bourgogne or F1 transgenic New Zealand females harboring a DNA construct with the coding sequence of the enhanced green fluorescent protein (EGFP) placed under the control of an elongation factor 1 (EF1) promoter. EGFP fluorescence and PCR amplification were used as markers of donor cumulus cells. These were kept at 38°C in Ca^{2+},Mg^{2+}-free PBS supplemented with 1% polyvinylpyrrolidone (PVP) 40,000 (Sigma) before being used as a source of nuclei.

Oocyte activation and nuclear transfer. To reconstruct NT embryos, individual cumulus cells were inserted by micromanipulation under the zona pellucida of the enucleated oocytes. NT embryos and MII oocytes were activated 18–20 h post-hCG as follows. Two sets of electrical stimulation were applied 1 h apart with a BTX stimulator (Biotechnologies & Experimental Research Inc., San Diego, CA) (3 DC pulses of 3.2 kV/cm for 20 µs each in mannitol 0.3 M in water containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$). The first set induced the cumulus cell–oocyte fusion. Reconstructed em-bryos and oocytes were then incubated for 1 h in M199 at 38°C. Then, the second set of pulses was applied to induce activation. NT embryos and oocytes were incubated for 1 h at 38°C in M199 containing 5 µg/ml CHX (Sigma) and 2 mM 6-DMAP (Sigma), then ret-urned to culture in a 50 µl microdrop of B2 medium (Laboratoire CCD, Paris, France) supplemented with 2.5% FCS under mineral oil (catalog no. M8410; Sigma) at 38°C under 5% CO_2 in air.

Analysis of preimplantation stages. Microtubule organization and chromatin in one-cell NT embryos were observed as already described[19], except that fixation lasted 20 min at 37°C and the mounting medium was Vectashield (Vector Laboratories, Burlingame, CA). Development rates until blastocyst stage were assessed after *in vitro* culture for 3 and 4 days. For cell number evaluation, embryos were fixed as above, stained with Hoechst 33342 at a concentration of 1 µg/ml, then mounted on well slides in Vectashield and monitored under epifluorescence.

Analysis of peri-implantation stages and *in vivo* development. Recipient females were mated to vasectomized males either at the same time (synchronous recipients), or 16 h or 22 h after the oocyte donors (asynchronous recipients). NT embryos were transplanted surgically through the infundibulum into each oviduct of recipients either at the one-cell stage (1–3 h post-activation) or at the four-cell stage (after an overnight culture). Implantation rate was assessed after killing of recipients at day 8 (D8). When visible, embryonic disks of blastocysts were measured microscopically (160×). Pregnancy was determined by palpation 13 or 14 days after embryo transplantation and the pregnant recipients delivered by caesarian section at 31 days post mating.

PCR analysis. The presence of the GFP transgenic marker was detected by PCR using a sense (5′-GAGTTTG-GATCTTGGTTCAT-3′) and an antisense (5′-GGCACGGGCAGCTTGCCGGTGG-3′) primer (Genset, Paris, France). To control the DNA quality, PCR was performed on 300–400 ng of DNA prepared with tissue extraction kit (Qiagen, Valencia, CA) with the sense primer, 5′-TTTTCCTGGATCATGCCTGGCAC-3′, and the antisense primer, 5′-CTACCTGTAGCAGCTTACCCA-3′, covering the exon 10 of the rabbit *CFTR* gene (Genset). Negative controls were double-distilled water and recipient female DNA, while positive controls were DNA from transgenic cultured fibroblasts.

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Competing interests statement

The authors declare that they have no competing financial interests.

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indicate a distribution of the presumptive auxin carriers part way up the sides of the cells in addition to the base would easily allow a lateral component to auxin transport if either only one side's carriers were activated or if basal and "other side" carriers were inhibited. Such differential activation of the auxin anion carriers is a concept already being incorporated into theoretical models of auxin transport in, for example, the geotropic response of stems (*21*).

The association of the labeled cells with vascular bundles in pea stems appears to be in general agreement with recent evidence from peas (*6*) that indicated a preferred auxin transport pathway within the vascular cylinder, specifically in cambium, procambium, and phloem initial cells. At this point, however, it is impossible for us to exclude the possibility that differential fluorescent labeling of cell and tissue types within our sections is due solely to such factors as differential penetration of cell walls by the immunoglobulin G antibody or a concentration of NPA binding sites in nonlabeled cells (or nonlabeled parts of basally labeled cells) that is too low to allow the development of a clear fluorescent signal. These alternatives deserve further investigation.

To our knowledge, this is the first report of monoclonal antibodies used to label plant tissue. Such antibodies are useful in that they can recognize a single antigenic site. Thus, one need not have a purified protein in order to obtain antibodies which specifically bind to it. We have obtained monoclonal antibodies that recognize an antigenic determinant of the NPA receptor in peas and have used them to identify that receptor at the basal ends of a population of pea stem parenchyma cells. In view of previous evidence linking NPA action to auxin efflux from cells and our present finding that the NPA binding site recognized by the monoclonal antibody used in our fluorescent localization studies can interact with auxin, these results strongly suggest that the auxin anion carrier of the chemiosmotic hypothesis is located at the basal plasma membrane of transporting cells.

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Nuclear Transplantation in the Mouse Embryo by Microsurgery and Cell Fusion

Abstract. *Nuclear transplantation in the mouse embryo was achieved by using a method that combines microsurgical removal of the zygote pronuclei with the introduction of a donor nucleus by a virus-mediated cell fusion technique. Survival of embryos was greater than 90 percent in tests of this procedure. The embryos developed to term at a frequency not significantly different from that of nonmanipulated control embryos. Because nuclei and cytoplasm from genetically distinct inbred mouse strains can be efficiently interchanged, this procedure may be useful in characterizing possible cytoplasmic contributions to the embryonic and adult phenotype.*

Nuclear transplantation studies in the amphibian embryo have provided valuable information about the possible restriction of nuclear potential during development (*1*). Similar experiments in the mammalian embryo are hindered by the small size of the embryo and its sensitivity to microsurgical manipulation. Despite these obstacles, transplantation of donor nuclei obtained from inner cell mass cells was recently reported in the mouse embryo (*2*, *3*). In those experiments, however, many of the manipulated embryos were lost because the plasma membrane was disrupted by a micropipette. We report a nuclear transplantation technique that avoids this loss by not requiring penetration of the embryo's plasma membrane with a micropipette. When pronuclei from a second one-cell stage embryo served as the nuclear donor, almost all embryos receiving this material survived the procedure and developed to term at a frequency comparable to that of unmanipulated control embryos.

Mouse embryos were incubated before and during microsurgery in cytochalasin B (*2–5*) and Colcemid. The embryo was secured by a holding pipette and the zona pellucida was penetrated with an enucleation pipette (Fig. 1). Penetration of the plasma membrane was avoided and the pipette was advanced into the embryo at a point adjacent to a pronucleus. Upon aspiration, a small portion of ovum plasma membrane and surrounding cytoplasm was drawn into the pipette, followed by the pronucleus. The pipette, now containing an entire pronucleus, was then moved to a point adjacent to the second pronucleus and the latter was similarly aspirated. As the enucleation pipette was withdrawn, a cytoplasmic bridge could be seen extending from the pronuclei in the pipette to the embryo (Fig. 1A). With continued withdrawal of the pipette, this bridge stretched to a fine thread and pinched off (Fig. 1B). The pipette, which now contained the membrane-bound pronuclei (pronuclear karyoplast), was moved to a

Table 1. Efficiency of the nuclear transplantation technique.

Genotype	Enucleation*	Karyoplast injection†	Fusion‡
C3H/HeJ	24 of 26	24 of 24	23 of 24
C57BL/6J	35 of 35	34 of 35	34 of 34
ICR	11 of 12	10 of 11	10 of 10
Total (%)	70 of 73 (96)	68 of 70 (97)	67 of 68 (99)

*Number of embryos surviving microsurgical removal of both the male and female pronuclei per total number of embryos. †Number of pronuclear karyoplasts surviving injection into the perivitelline space of the recipient embryo per total number of karyoplasts injected. ‡Number of pronuclear karyoplasts fusing with the recipient embryo per total number of karyoplast-injected embryos.

second drop containing Sendai virus inactivated with β-propiolactone (2000 to 3000 hemagglutinating units per milliliter), and a small volume of virus suspension, approximately equal to the volume of the pronuclear karyoplast in the pipette, was aspirated. The pipette was then moved to a third drop containing a previously enucleated embryo. The zona pellucida of this embryo was penetrated at the previous site of enucleation, and the virus suspension and pronuclear karyoplast were injected sequentially into the perivitelline space (Fig. 1C) (*6*). The pipette was withdrawn and the embryo was incubated at 37°C. Fusion of the pronuclear karyoplast with the enucleated embryo usually occurred during the first hour of incubation (Fig. 1D).

Thus, with this procedure, it is possible to transfer the nuclei from one embryo to another without penetrating the plasma membrane. Furthermore, it becomes possible to use large-bore pipettes, which are necessary to accommodate pronuclei but are highly disruptive in existing mechanical microinjection techniques.

To determine the overall efficiency of this transplantation procedure and its effects on subsequent development, we transplanted pronuclei between genetically distinct one-celled mouse embryos and transferred to the uteri of pseudopregnant females those embryos that had developed to the blastocyst stage after 5 days in vitro. Control embryos isolated at the one-cell stage were similarly cultured and transferred to the uteri of pseudopregnant females, but were not exposed to cytoskeletal inhibitors or inactivated Sendai virus. Of 73 experimental embryos, 70 (96 percent) were successfully enucleated, and of the 70 pronuclear karyoplasts obtained, 68 (97 percent) were successfully introduced (with Sendai virus) into the perivitelline space of enucleated zygotes (Table 1). After incubation at 37°C, 67 (99 percent) of these karyoplasts fused to the plasma membrane of the ovum. The overall efficiency of nuclear transplantation was therefore 91 percent.

After microsurgery, experimental and control embryos were cultured for 5 days and the number of embryos successfully developing to the blastocyst stage was determined. Of 34 control embryos, all developed to the morula or blastocyst stage (Table 2). Similarly, of the 67 experimental embryos, 64 (96 percent) developed to the morula or blastocyst stage. Transfer of the 34 control embryos to the uteri of pseudopregnant females resulted in the birth of five progeny (15 percent), three of which survived to adulthood. Transfer of the 64 experimental embryos to the uteri of psuedopregnant females resulted in the birth of ten progeny (16 percent), seven of which survived to adulthood. These seven offspring all displayed the coat color phenotype of the donor nuclei, and five were fertile.

Thus the technical manipulations involved in transferring pronuclei from one zygote to another did not significantly affect the ability of embryos to undergo normal development. The high frequency of developmental arrest in both the experimental and control groups after the implantation procedure may have resulted from the in vitro culture period

Table 2. Development of control and nuclear-transplant embryos. The subscripts N and C refer to the strain origin of the nucleus and of the cytoplasm, respectively.

Group and strain	Developmental stage by day 5 in vitro (number of embryos)			Number born*
	Arrested	Morula	Blastocyst	
Control				
C3H/HeJ	0	3	11	0
C57BL/6J	0	1	13	4
ICR	0	1	5	1
Total	0	5	29	5
Nuclear-transplant				
$C3H/He_N$ to $C57BL/6J_C$	0	0	23	4
$C57BL/6J_N$ to $C3H/He_C$	0	1	23	3
ICR_N to $C57BL/6J_C$	1	0	9	2
$C57BL/6J_N$ to ICR_C	2	0	8	1
Total	3	1	63	10

*Number of offspring born after transfer of morulae and blastocysts into the uteri of females in the third day of pseudopregnancy.

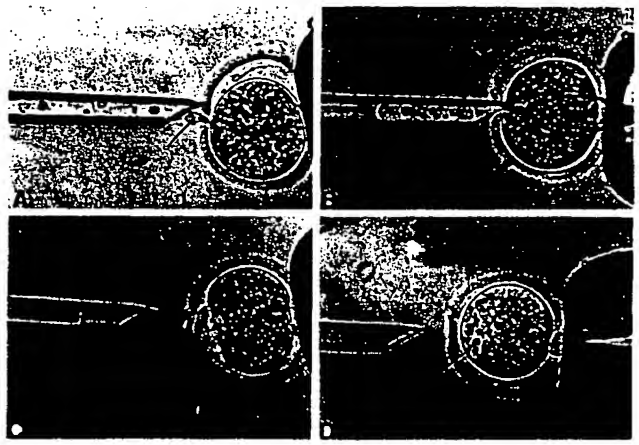

Fig. 1. Nuclear transplantation in the mouse embryo. One-cell stage embryos were obtained from oviducts excised from spontaneously mated females on the day of vaginal plug detection (day 1). Cumulus cells were dispersed in Whitten medium (*8*) containing bovine hyaluronidase (500 nonfiltered units per milliliter). The embryos were washed four times and cultured to the blastocyst stage in 50-μl drops of modified Whitten medium (*9*) under silicone oil at 37°C in an atmosphere of 5 percent O_2, 5 percent CO_2, and 90 percent N_2 (*10*). Medium containing cytochalasin B (5 μg/ml) and Colcemid (0.1 μg/ml) was prepared weekly, stored at 4°C, and protected from light. Microsurgery was performed with Leitz micromanipulators and a fixed-stage Leitz Laborlux II microscope. Holding and enucleation pipettes were fashioned from Pyrex capillary tubing (outer diameter, 1.0 mm; inner diameter, 0.65 mm). For holding pipettes (outer diameter, 75 to 100 μm), the capillary tubing was hand-pulled over a microburner, placed on a DeFonbrune microforge, broken on a glass anvil, and polished. Enucleation pipettes (outer diameter, 15 to 20 μm) were fashioned with a DKI 200 vertical pipette puller and their tips were beveled on a grinding wheel, dissolved in a solution of hydrofluoric acid (25 percent), and sharpened on a microforge. They were then treated with 100 percent Nonidet P40, rinsed thoroughly, and stored overnight. Before microsurgery, groups of six to eight embryos were incubated for 15 to 45 minutes at 37°C in an atmosphere of 5 percent O_2, 5 percent CO_2, and 90 percent N_2 in bicarbonate-buffered Whitten medium containing cytochalasin B and Colcemid. They were then placed singly in hanging drops of Hepes-buffered Whitten medium containing cytochalasin B (5 μg/ml) and Colcemid (0.1 μg/ml) in a Leitz oil chamber. Microsurgery was performed as described in text, and the embryos were washed and returned to the incubator. All microsurgery was performed at room temperature. Sendai virus was obtained from the infected allantoic fluid of embryonated chicken eggs and inactivated with β-propiolactone (*11–13*). (A) Light micrograph of an embryo in the process of enucleation. Note the pronuclei in the pipette and the cytoplasmic bridge (arrow) between the cytoplasm of the embryo and the pronuclear karyoplast. (B) Light micrograph of an enucleated embryo. The pipette contains the pronuclei surrounded by a small volume of cytoplasm and a portion of the embryo's plasma membrane. (C) Light micrograph of an enucleated embryo after introduction of inactivated Sendai virus and the pronuclear karyoplast into the perivitelline space. (D) Light micrograph of an embryo shortly after fusion of the pronuclear karyoplast to the enucleated embryo. Note the peripheral location of the pronuclei (arrow).

before implantation, since the intrauterine transfer of 22 carrier blastocysts that had developed in vivo resulted in the birth of 17 progeny. The method described here can also be used to introduce donor nuclei obtained from later-stage embryonic cells into enucleated zygotes (*7*). This procedure may therefore aid in further defining the possible developmental restriction of nuclei during mammalian embryogenesis. In addition, reciprocal pronuclear transplantations between genetically distinct one-celled embryos may be used to define the degree to which maternally inherited cytoplasmic components persist.

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Relative Brain Size and Metabolism in Mammals

Abstract. *Comparisons of the relation between brain and body weights among extant mammals show that brain sizes have not increased as much as body sizes. Interspecific increases in brain and body size appear to occur at the same rate, however, when the amount of available energy is taken into account. After this adjustment, brains of primates are slightly larger than expected from the overall mammalian data, but primates also use a larger proportion of their total energy reserves for their brains. Analyses of relative brain size must take into account the requirements that the metabolically active brain has for the body.*

For the past 50 years the relation of brain to body weights among different mammalian taxonomic groups has been thought to scale allometrically at 0.67 (*1*, *2*), but recent expansion of the data base led to estimates of the slope being approximately 0.75 (*3*). The newer and larger sets of points may have disproportionately increased the numbers of small mammals with relatively small brains and this alone could produce a steeper slope (*4*). Although the reason for the discrepancy between the slopes is not known, the mammalian data sets appear regular and contain certain consistent deviations; anthropoids (*1–3*, *5*), pinnipeds, and odontocetes (*5*) are highly encephalized and frugivorous bats are more encephalized than insectivorous ones (*6*), a situation that may have parallels among primates (*7*).

The causal factors controlling brain to body-weight scaling are not known, but it has been conjectured that the scaling reflects the functions of the brain for analyzing sensory information and controlling motor output (*2*, *8*). The brain controls the body's actions but also needs the body for its energy supply. The brain is metabolically very active and demands a large supply of oxygen and glucose, as much during sleep (*9–11*) as during increased mental activity (*9*, *11*). Regulation of cerebral homeostasis permits small perturbations in the delivery of oxygen and glucose, but decreased availability of oxygen or glucose are associated with pathological states such as coma (*9*, *10*, *12*). The metabolic relation between the brain and body has received attention (*3*, *13*, *14*), but its role in relative brain size has not been adequately analyzed (*15*). It is proposed here that the size of the brain will be constrained both by the size of the system delivering oxygen and glucose and by the rate at which energy can be expended on supporting the brain's constantly high metabolic demands. Body weight is a first approximation of the size of the storage and delivery systems for glucose and oxygen, and the organism's basal (standard) metabolic rate (BMR) estimates the amount of available oxygen and energy per unit time (*16*).

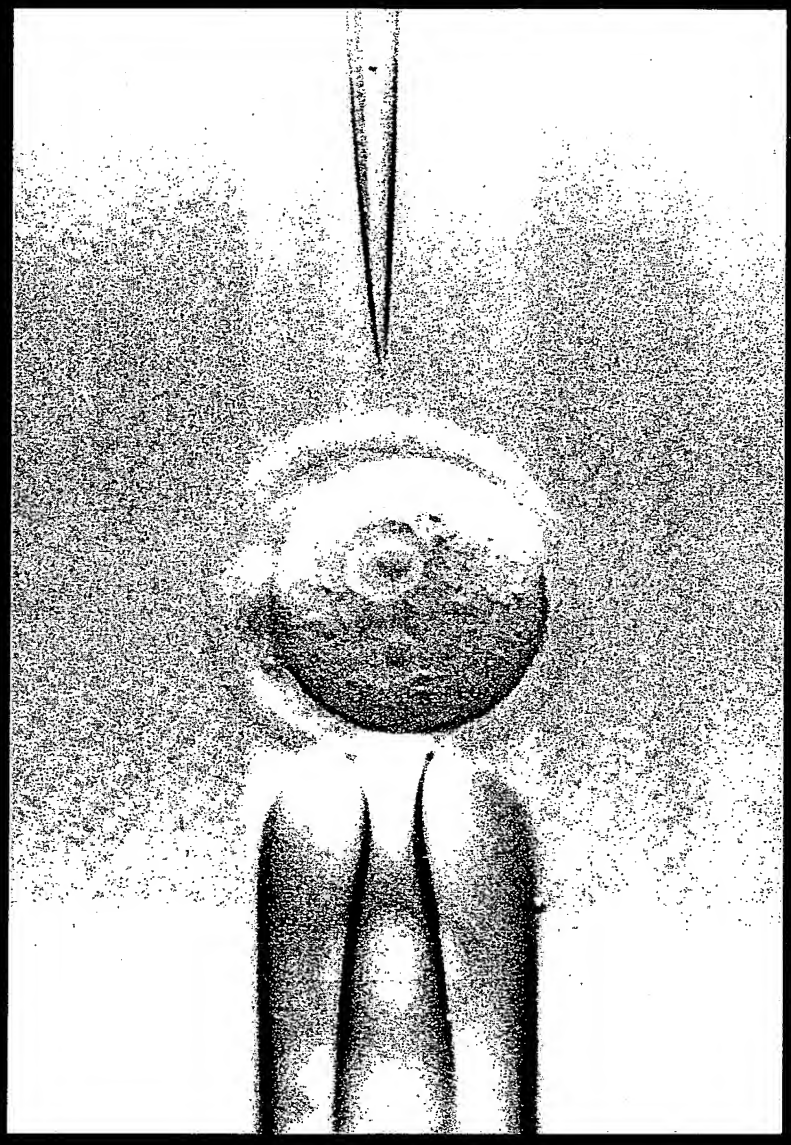
In this study brain weights of 93 adult mammalian species were collected from the literature (*1–3*, *6*, *13*, *17*) and analyzed allometrically in terms of both body weight and body mass times the metabolic rate. These adjusted body weights parallel the animal's caloric expenditure. Only species that had brain weights, body weights, and BMR's (in cubic centimeters of O_2 per 100 g per minute) were used. If the studies in which the species-specific brain weight and BMR were determined used individuals of a species whose body weights differed by more than 10 percent, the BMR was adjusted (*13*). Rates of total brain metabolism measured with the Kety-Schmidt technique were also taken from the literature (*13*). Linear regressions and principal axes were used to study the relation among the logarithmically transformed data. Comparisons of intercepts or adjusted group means are based on analyses of covariance ($N - 3$ degrees of freedom) and reported as t-tests (Table 1).

The overall picture of the regression of brain weights against body weights resembles other mouse-to-elephant curves (Fig. 1). The relation is best described by the linear regression equation $\log E = -1.28 + 0.76 \log S$ [$r = .976$, 95 percent confidence limits of the slope (cls) = 0.743 to 0.779], where E = brain weight and S = body weight; (slope of the principal axis = 0.761). The slope from these data is very close to recent estimates (*3*) and higher than the 0.67 slopes reported earlier (*1*, *2*, *5*). Although primates ($\log E = -1.11 + 0.81 \log S$; 95 percent cls = 0.693 to 0.927; $r = .973$) have larger relative brains compared to all other mammals ($\log E = -1.29 + 0.74 \log S$; 95 percent cls = 0.707 to 0.776; $r = .983$), the pinnipeds and odontocetes have relatively big brains with values overlapping those of large anthropoids (*18*). Furthermore, the pinnipeds and odontocetes have larger relative brain sizes than do terrestrial ungulates (artiodactyls, perissodactyls, and elephant). Frugivorous bats have bigger brains per body weight (*19*) than insectivorous bats, corroborating earlier reports (*6*, *7*) (Table 1). Because only one insectivorous primate, *Galago demidovii* (*20*), was included in this sample, statistical analyses were not run on dietary differences among primates.

For this mammalian sample, the amount of O_2 consumed per body weight is described by the equation $\log \text{BMR} = 0.84 - 0.269 \log S$ (95 percent cls = −0.292 to −0.248; $r = -.93$; principal axis slope = −0.270), and the slope is close to the predicted −0.25 one (*21*). The unexplained variance for BMR to body weight is higher than that for brain to body weight, reflecting either an increase in measurement error or a larger biological variation. Several taxonomic deviations from the overall trend occur here too. Primates (log BMR = 0.60 −

Manipulating the Mouse Embryo

A LABORATORY MANUAL



Brigid Hogan
Frank Costantini
Elizabeth Lacy

Manipulating the Mouse Embryo

A LABORATORY MANUAL

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Cold Spring Harbor Laboratory
1986

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EMBRYO TRANSFERS

Embryos from the one-cell stage through to the blastocyst stage (0.5–3.5 days p.c.) can be transferred into the reproductive tract of a pseudopregnant recipient to complete their development. Embryos from the one-cell through the morula stage (0.5–2.5 days p.c.) are transferred into the ampullae of 0.5-day p.c. pseudopregnant recipients, whereas 3.5-day blastocysts are transferred into the uterine horns of a 2.5-day p.c. (or at the very latest a 3.5 day p.c.) pseudopregnant recipient. The former procedure is called an oviduct transfer and can only be performed with embryos enclosed within a zona pellucida. The latter procedure is referred to as a uterine transfer and for this type of transfer the embryos need not have a zona pellucida. The reason for transferring embryos into a female at an earlier age of pseudopregnancy is to give the embryo time to "catch up" in its development before being exposed to conditions favorable for implantation. In general, for both procedures, 50–75% of unmanipulated embryos will develop to term. Ideally, enough embryos should be transferred to give a litter size of five to seven. If there are only one or two embryos in the uterus they may grow too big to be born without being damaged. Also, mothers may not take care of small litters. If the litters are too large (more than 10) then a few may grow up small, with a risk of being sterile. Provided that they have been given embryos injected with the same DNA, two foster mothers can be placed in the same cage after the transfer operation. They will subsequently help each other bring up a joint litter.

Oviduct Transfer

EQUIPMENT

Avertin (anesthetic)
Animal balance
1-cc syringe
26-gauge, 1/2-in. needle
Animal clippers (optional)
70% ethanol
Tissues (several rolled into small swabs are useful for soaking up any blood)
Dissection scissors
Two #5 watchmaker's forceps (clean and sharp)
One blunt fine forceps
Surgical silk (size 5-0)
Curved surgical needle (e.g., size 10, triangular, pointed)
Serafine (1.5 in. or smaller)
Two steromicroscopes ideally (one for the operation, one for loading the embryo transfer pipet)
Fiber optic illuminator
Transfer pipet and mouth pipet
Alcohol burner
Wound clips and clip applicator
Lid of 9-cm plastic petri dish or glass plate

The recipients should be females, at least 6 weeks of age and >20 g in weight, mated to vasectomized males the evening before the transfer. Therefore, the females will be approximately 0.5 day p.c. pregnant at the time of the transfer. F_1 hybrid (e.g., C57BL/6×CBA or C57BL/6×DBA) or outbred females, such as Swiss albino mice, make good foster mothers. It is a good idea to have excess pseudopregnant females available in case the dissection of the oviducts is unsuccessful.

PROCEDURE

It is best to practice this procedure on a cadaver, and to inject a dye solution rather than eggs to convince yourself that you can find the opening to the oviduct. It is useful to remember that the position of the infundibulum is relatively invariant from mouse to mouse; with a little practice the technique will become routine and will cause minimal distress to the mouse.

1. Anesthesize the recipient. Weigh the recipient mouse and inject it intraperitoneally with 0.015–0.017 ml of 2.5% Avertin (Section H) per gram of body weight. (The proper dose of Avertin may vary with different preparations, and should be redetermined each time a new stock is prepared.) Place the mouse on a lid of a petri dish (or similar item) so that it can be lifted onto the microscope stage easily.

2. Shave the lower back of the recipient mouse (optional).

3. Load a transfer pipet with embryos. Since they will be outside the incubator for several minutes, transfer any embryos in M16 into M2 before loading. Transfer pipets are pulled in advance from BDH hard glass capillary tubes. The narrow part of the pipet should be 2–3 cm in length and 120–180 μm in diameter, i.e., just larger than one embryo and smaller than two. Also, the tip should be flush, and should be flame-polished, in order to minimize damage to the oviduct. The pipet is first filled with light paraffin oil to just past the shoulder (Fig. 51). The viscosity of the oil allows one to pick up or blow out the embryos with greater control. As an alternative to using oil, the other end of the pipet can be melted down to a narrow opening (see Implantating Tissues under the Kidney Capsule).

   A small amount of air is taken up, then medium M2, and then a second air bubble. Next the embryos are drawn up in a minimal volume of medium, then a third air bubble is taken up, followed by a short column of medium (see Fig. 51). Store the transfer pipet (still in the mouth-pipeting device) by pressing it into a piece of plasticene stuck to the stereomicroscope, and leave it there until you are ready to place the embryos in the oviduct. BE CAREFUL NOT TO DISTURB THE PIPET.

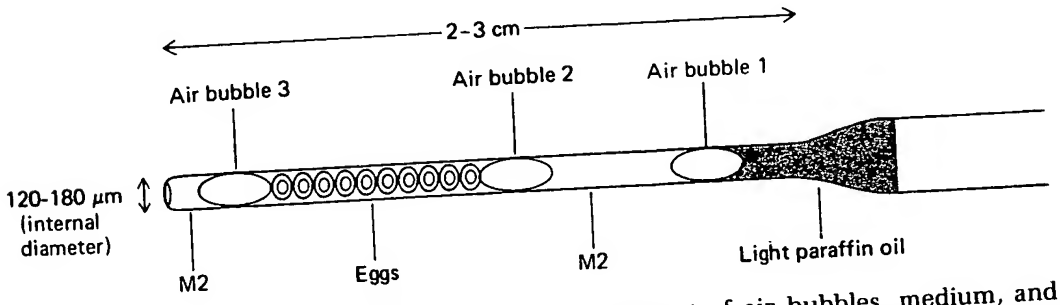


Figure 51 Embryo transfer pipet showing arrangement of air bubbles, medium, and embryos. Monitoring the position of the air bubbles enables the operator to be sure that all of the embryos have been injected.

4. Transfer the embryos.

   a. Sterilize all instruments by dipping them in 100% ethanol and flaming them with the alcohol burner. After wiping the mouse's back with 70% ethanol, make a small transverse incision (less than 1 cm) with the dissecting scissors, about 1 cm to the left of the spinal cord, at the level of the last rib (Fig. 52). Wipe the incision with 70% alchohol to remove any loose hairs.

   b. Slide the skin around until the incision is over the ovary (orange) or fat pad (white), both of which are visible through the body wall. Then pick up the body wall with the watchmaker's forceps and make a small incision just over the ovary. Stretch the incision with the scissors to stop any bleeding. With a surgical needle, thread a piece of silk suture through the body wall so that the body wall will be easy to locate later. With the blunt forceps, pick up the fat pad and pull out the left ovary, oviduct, and uterus, which will be attached to the fat pad. Clip the serafine onto the fat pad and lay it down over the middle of the back, so that the oviduct and ovary remain outside the body wall.

   c. Gently pick up the mouse and place it with head to the left on the stage of the stereomicroscope. This procedure is easier if the mouse is laid out initially on the lid of a petri dish or on a glass plate.

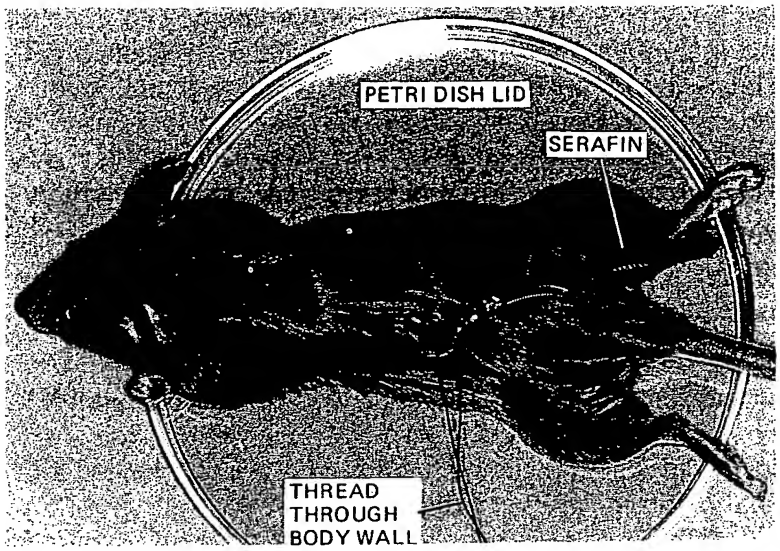


Figure 52 Mouse prepared for oviduct transfer. (*A*) The anesthetized mouse is placed on a petri dish lid for ease of handling. Ovary, oviduct, and proximal end of the uterus are held outside the body by means of a serafine clip attached to the fat pad above the ovary. The incision in the body wall is located with a suture thread. (*B*) The ovary/oviduct/uterus are held outside the body cavity by a serafine attached to the fat pad above the ovary. The orientation shown here gives easy access to the oviduct for transfer of embryos by the technique described in the text. After returning the uterus, etc., to the body cavity, the incision is located and closed by the thread through the body wall. In this demonstration the hair around the incision has not been shaved; this is an optional step that may be helpful when carrying out the procedure for the first few times.

d. Under the stereomicroscope locate the opening (infundibulum) to the oviduct and the swollen ampulla underneath the bursa (a transparent membrane over the oviduct and ovary). Arrange the mouse, oviduct, etc., so that the pipet can enter easily (Fig. 53). It is most convenient to have the head to the left and the ovary, etc., pulled to the rear with the serafine. With two watchmaker's forceps, tear a hole in the bursa over the infundibulum. Be careful not to tear through any large blood vessels.

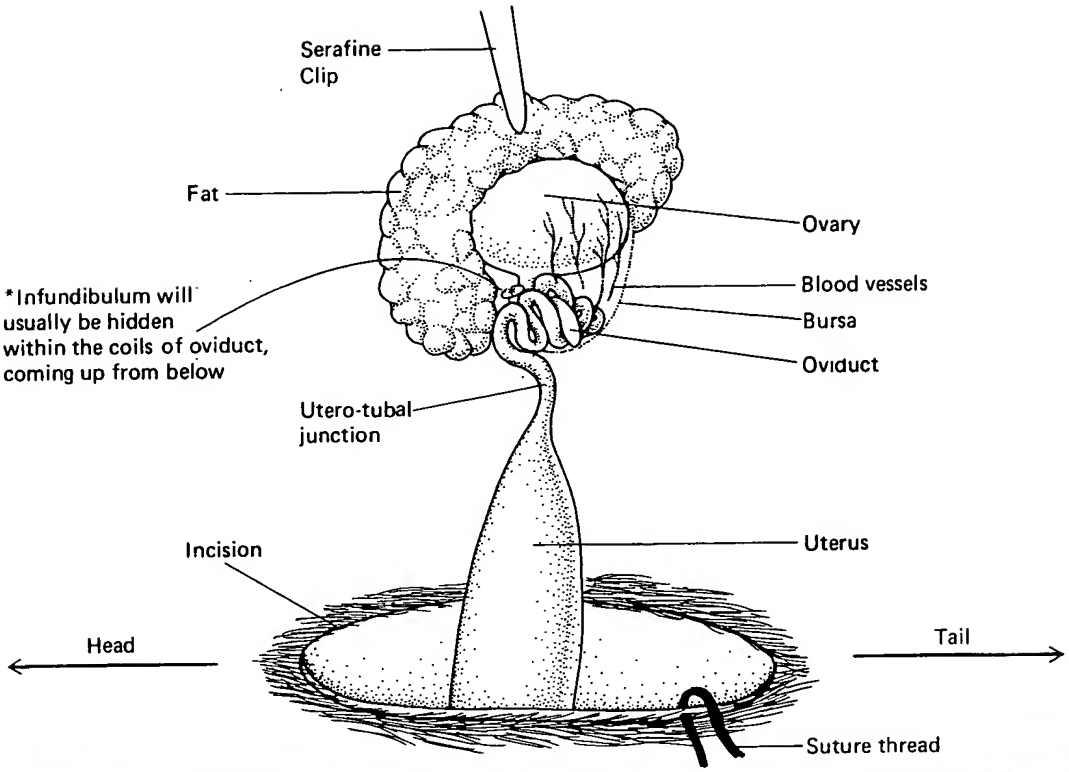
e. Pick up an edge of the infundibulum or the bursa near the infundibulum with fine forceps and then insert the pipet down the opening to the ampulla. Blow until both air bubbles 2 and 3 have entered the ampulla.

f. Unclip the serafine and remove the mouse from the stereomicroscope. With the blunt forceps, pick up the fat pad and push the uterus, oviduct, and ovary back inside the body wall. Sew up the body wall with one or two stitches (optional) and close the skin up with wound clips.

5. Repeat steps 3 and 4 to transfer additional embryos to the right oviduct, if desired.

6. At the end of the operation, return the mouse to its cage and leave undisturbed in a warm, quiet place. It should recover from the anesthetic in about 20–30 minutes.

Rafferty (1970) and Dickman (1971) describe slightly different procedures for oviduct transfer.



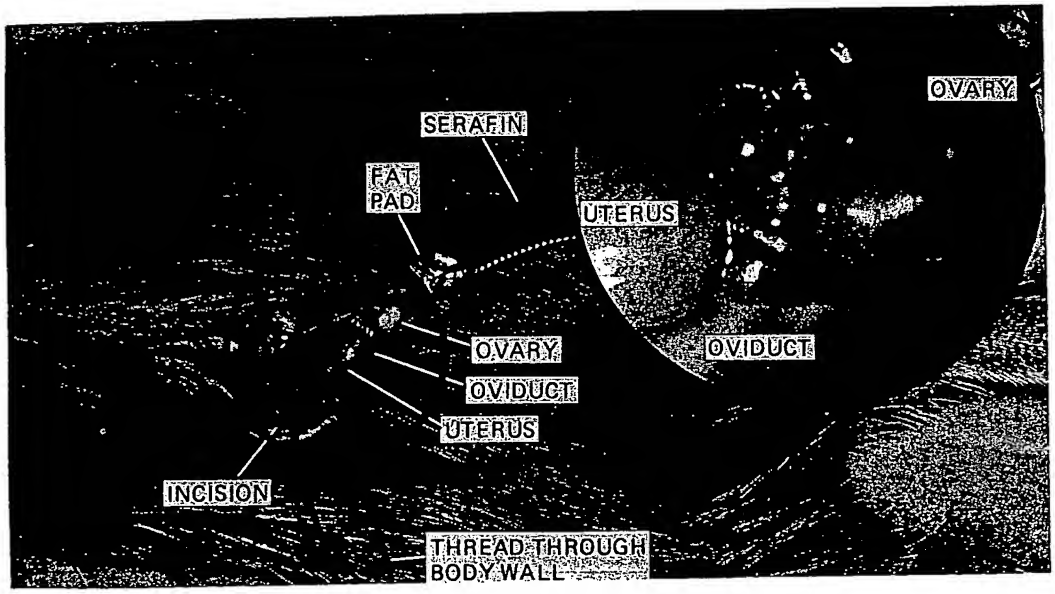


Figure 53 Detail for oviduct transfer. The oviduct is surrounded by a thin transparent bursa or membrane containing blood vessels. Make a small incision in the membrane (avoiding blood vessels), locate the end of the oviduct (infundibulum), and hold the edge with fine forceps while inserting the tip of the transfer pipet.